Kinetics of Binding of Oligosaccharides to a Homogeneous Pneumococcal Antibody: Dependence on Antigen Chain Length Suggests a Labile Intermediate Complex†

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ABSTRACT: Temperature-jump experiments were performed with di-, tetra-, and hexasaccharides derived from type III pneumococcal polysaccharide using a homogeneous corresponding antibody IgG 45-394. A decrease in stability of the oligosaccharide–antibody complexes with decreasing chain length was observed and was entirely reflected in the decrease of the association rate constants which were 1.7×10^4 M^{-1} s^{-1} for the di-, 3.7×10^5 M^{-1} s^{-1} for the tetra-, and 1.1×10^6 M^{-1} s^{-1} for the hexasaccharide at 23 °C. The dissociation rate constants for all oligomers were about 12 s^{-1}. This marked chain-length dependence of the association rate constants as well as their low values are unexpected for a single binding step. A mechanism is proposed which consists of a fast formation of a labile oligosaccharide–antibody precomplex followed by a slow isomerization step which is induced by the oligosaccharide ligands but which is chain-length independent.

Recent evidence for structural transitions in homogeneous antibody molecules upon antigen binding has been shown by circular dichroism (Holowka et al., 1972; Jaton et al., 1975a) or circular polarization of luminescence (Schlessinger et al., 1975a; Jaton et al., 1975b). It is reasonable to assume that the manifestation of such transitions should be apparent in the initial binding steps of antigen recognition by antibody. Most of the kinetic studies to date involved binding of small haptenic determinants to either heterogeneous or homogeneous antibodies (Froese and Sehon, 1975; Haselkorn et al., 1974). Recent x-ray diffraction studies (Poljak, 1975; Davies et al., 1975) have suggested that these small ligands are likely to occupy a relatively small portion of the potentially large binding area in the antibody combining site, and therefore the pathway of binding in these cases might not be the same as that expected for protein or polysaccharide antigens in combination with their antibodies. In this connection, it is noteworthy that the initial rate of reaction of antibody with a protein antigen appeared to be at least 100 times slower than a typical antibody–hapten reaction (Dandliker and Levison, 1967; Levison et al., 1971), a fact which is compatible with the possible occurrence of conformational changes during binding reaction.

The availability of monoclonal rabbit antibodies to type III pneumococci and a series of oligosaccharides of increasing size derived from SIII polysaccharide offers the advantage that the antibody is homogeneous and that the hexasaccharide exhibits the optimal size which fits into the combining site (Mage and Kabat, 1963; Davies and Padlan, 1977). Results of kinetic studies of the binding of di-, tetra-, and hexasaccharides to a specific rabbit antibody are presented.

Materials and Methods

The homogeneous rabbit antibody 45-394 directed against type III pneumococcal polysaccharide was raised according to the method of Kimball et al. (1971) and purified as described previously (Braun and Jaton, 1973). Fab[1] fragment was prepared by papain digestion from antibody 45-394 by the method of Porter (1959). Antibody concentration was determined spectrophotometrically at 279 nm using $E_{1\%} = 14.5$ in 0.2 M phosphate buffer, pH 7.2, containing 0.02% sodium azide. All measurements were performed in this buffer. Di-, tetra-, and hexasaccharides derived by partial acid hydrolysis from SIII pneumococcal polysaccharide were obtained as described by Campbell and Pappenheimer (1966). Solutions of oligosaccharides at suitable molar concentrations were prepared from salt-free lyophilized preparations using a molecular weight of 370 for the disaccharide unit.

Temperature-jump experiments were performed on an apparatus constructed by Dr. G. Hänisch, Department of Biophysical Chemistry, Biocenter, according to the design of Rigler et al. (1974). A discharge voltage of 25 kV produced a temperature jump of about 3 °C. The temperature before discharge was adjusted by an external water bath to various temperatures (±0.1 °C). The difference between the temperature inside the cell and the external water bath was determined by a thermistor which was placed in the cell. For this calibration the cell was filled with buffer. The kinetics was monitored by the change in protein fluorescence which accompanies antigen binding (Jaton et al., 1975a). The excitation wavelength was 280 nm and fluoresecence emission was measured at wavelengths above 305 nm using a cut-off filter. The data were digitized by a Datalab DL 905 transient recorder and processed by a PDP 11-40 computer (Digital Equipment Co.). Depending on the signal-to-noise ratio, the data of 6 to 12 experiments were averaged. A program developed by Dr. Carlton Paul, Department of Biophysical Chemistry, Biocenter, was employed. The relaxation times were evaluated from the averaged single exponential curves either by the computer or manually. At the excitation wavelength of 280 nm very small absorption changes were observed on hapten binding. Therefore the kinetically observed changes in fluorescence are linearly related to concentration changes.

Results

Temperature-jump experiments were performed using

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[1] Abbreviation used: Fab, monovalent antigen binding fragment.

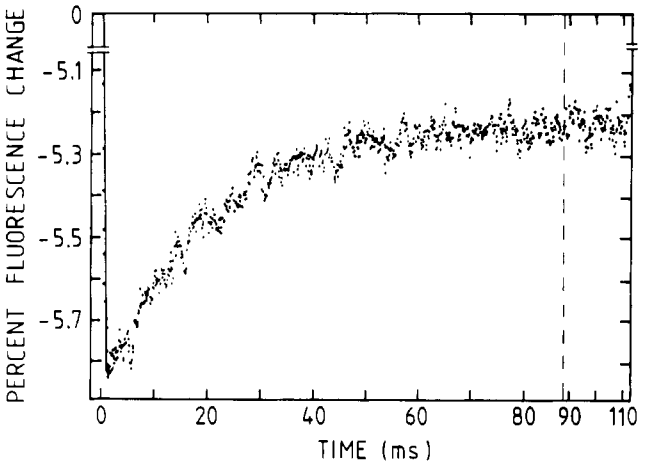

FIGURE 1: Time course of fluorescence change for IgG with hexasaccharide after a temperature jump of 3 °C. IgG concentration was 1×10^{-6} M and hexasaccharide concentration was 2.9×10^{-5} M. The temperature before the temperature jump was 26.5 °C. The rise time as determined by the electronic filter was 0.5 ms. The figure represents an original computer output obtained by averaging 13 experiments. The vertical broken line indicates a change in time scale.

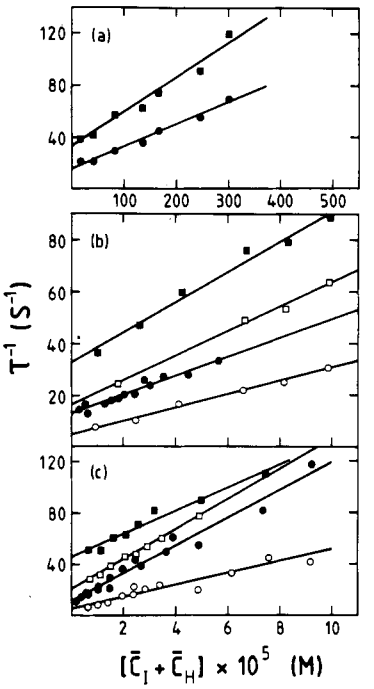

FIGURE 2: Concentration dependence of the reciprocal relaxation times for the binding of oligosaccharides to IgG. The τ^{-1} values are plotted as a function of the sum of the concentrations of free binding sites ($\bar{c}_A$) and of the free haptens ($\bar{c}_H$) at various final temperatures. (a) IgG with disaccharide at 23.3 °C (●) and 35 °C (■). (b) IgG with tetrasaccharide at 16 (○), 23.3 (●), 28.3 (□), and 35 °C (■). (c) IgG with hexasaccharide at 14.5 (○), 23.3 (●), 29.3 (□), and 35 °C (■). Lines represent least-square fits to the data.

antibody IgG with di-, tetra-, and hexasaccharide ligands. An initial large decrease of fluorescence occurred within 5 μs which is close to the time resolution of the instrument. This time of 5 μs was derived from temperature-jump experiments using an electronic filter with a rise time 500 times shorter than that used for the experiment shown in Figure 1. The first unresolved phase was followed by a kinetically resolved increase of fluorescence (Figure 1). In all experiments single exponential curves were observed. The relaxation times varied from 10 ms to several 100 ms depending on concentration, temperature, and the nature of the hapten. IgG in the absence of hapten exhibited the first unresolved fluorescence change only.

The reciprocal relaxation time (τ^{-1}) increased with increasing concentrations of the reaction partners in the way expected for a second-order reaction. In Figure 2, τ^{-1} is plotted vs. the sum of the free binding site concentration of antibody ($\bar{c}_A$) and hapten ($\bar{c}_H$) according to eq 1 at various temperatures.

$$\tau^{-1} = k_a(\bar{c}_H + \bar{c}_A) + k_d \qquad (1)$$

In eq 1 k_a and k_d are the rate constants of association and dissociation respectively. For all measurements with the disaccharide (Figure 2a), the hapten to binding site ratio was about 1000. In this case the sum of the free concentrations was approximated by the total hapten concentration (c_H). The same approximation was applied when tetra- and hexasaccharides (Figures 2b and 2c) were used at c_H larger than 10^{-5} M for which the molar ratio of hapten to binding sites was larger than 5. For c_H smaller than 10^{-5} M, the free concentrations were calculated from the total concentrations and the equilibrium binding constant obtained from the rate constants (see below). The rate constants of dissociation and association were derived from the intercepts and the slopes of the lines in Figure 2, respectively, according to eq 1. The data measured at 23 °C computed from Figure 2 are summarized in Table I. The binding constants calculated from the rate constants agree with those previously determined by equilibrium methods (Jaton et al., 1975a). The dependence of the amplitude of the relaxation process on hexasaccharide concentration at constant IgG concentration was determined and was consistent with a second-order process and a binding constant of 9×10^4 M^{-1}. The increase of stability of the antibody–oligosaccharide

TABLE I: Rate Constants and Equilibrium Constant for the Binding of Oligosaccharides to Antibody 45-394 and Its Fab Fragment at 23 °C.

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K^a (M^{-1})	K^b (M^{-1})
IgG				
Disaccharide	1.7×10^4	16	1.1×10^3	
Tetrasaccharide	3.7×10^5	13	2.9×10^4	
Hexasaccharide	1.1×10^6	12	9×10^4 (9×10^4)	$(1.4 \pm 1) \times 10^5$
Octasaccharide				$(3.0 \pm 1) \times 10^5$
Fab				
Hexasaccharide	8.3×10^5	11	7.7×10^4	$(2.4 \pm 1) \times 10^5$

[a] The binding constant K was calculated from the association rate constant k_a and the dissociation rate constant k_d (estimated error for the rate constants ±20%). The value for K in parentheses was derived from the concentration dependence of the amplitude of the kinetic phase. [b] Values determined by fluorescence titration (Jaton et al., 1975a).

complex with increasing chain length of the saccharides is in agreement with similar observations for other immunoglobulins directed toward oligosaccharides (Cisar et al., 1975). The leveling-off of the chain-length dependence of the binding constants shown in Table I suggests that the binding site of IgG 45-394 may accommodate about six saccharide units. This is in accordance with fluorescence titration studies on the hexa- and octasaccharide (Jaton et al., 1975a) and with the size of the binding site evaluated on the basis of similar arguments (Holowka et al., 1972; Cisar et al., 1975). There is a striking

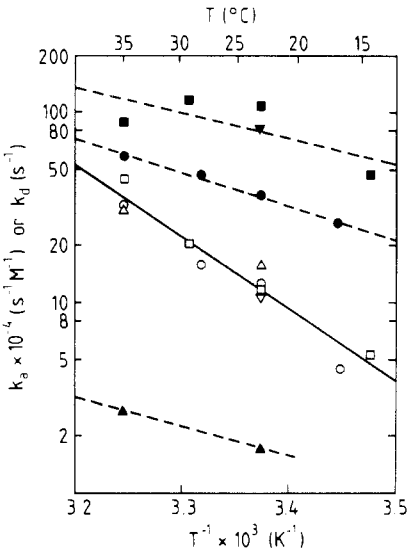

FIGURE 3: Arrhenius plot of the association and dissociation rate constants. Solid symbols indicate association rate constants and open symbols dissociation rate constants. Results for antibody IgG and disaccharide (△), tetrasaccharide (○), hexasaccharide (□), and Fab fragment and hexasaccharide (▽) are plotted. The solid line represents a least-square fit to the dissociation rate constants obtained for all oligosaccharides. The association rate constants were fitted separately for each oligosaccharide (dashed lines).

increase in the association rate constant with increasing chain length; the increase in k_a parallels that of the binding constant, whereas the dissociation rate constant is independent of chain length (Table I).

The Arrhenius plots of the temperature dependence of k_d and k_a are shown in Figure 3. The dissociation rate constants fall on one line corresponding to an activation energy of 75 ± 5 kJ/mol regardless of the chain length of the oligosaccharides. The data for the association rate constants differ markedly for the three oligosaccharides but the slopes of the Arrhenius plots do not differ significantly. For the tetrasaccharide, the activation energy was found to be 33 ± 5 kJ/mol. The value for the disaccharide as judged from only two points was very similar (30 ± 5 kJ/mol). The value obtained by a least-square fit to the scattered data for the hexasaccharide (25 ± 10 kJ/mol) is not significantly different. From $\Delta H° = E$(association) − E(dissociation), the enthalpy of the overall process may be estimated to be −45 ± 10 kJ/mol. It is not possible to establish a clear chain-length dependence of $\Delta H°$. Due to the large temperature dependence of the fluorescence signal, only the sign of $\Delta H°$ was confirmed by equilibrium measurements. Since the fluorescence decreases on complex formation (Jaton et al., 1975a), the increase of fluorescence in the kinetically resolved phase following an increase in temperature (Figure 1) proves that $\Delta H°$ is negative. The entropy of the overall process is about $\Delta S° = -(60 \pm 15)$ J mol^{-1} K^{-1}. Although the chain-length dependence of $\Delta G°$ is clearly established (Figure 2), the chain-length dependence of $\Delta S°$ is uncertain because of the large error limits of $\Delta H°$.

Discussion

The most striking result of the present study is that the association rate constants increase with increasing chain length of the oligosaccharides, whereas the dissociation rate constants stay essentially constant (Table I). This contrasts with the normal behavior in complex formation for which an increase in stability of the products due to chemical modifications of the reaction partners is reflected by a decrease of the dissociation rate constant whereas the association rate constant (k_a) changes usually much less. Only small changes of k_a in a homologous series of reactants are expected on theoretical grounds for diffusion-controlled processes (Eigen et al., 1964; Amdur and Hammes, 1966). Such a constancy is also observed for binding processes which are slower than diffusion controlled. These normal features of association reactions are demonstrated for IgG-hapten system by the reaction of myeloma proteins with DNP derivatives (Haselkorn et al., 1974) and by other protein systems (Lazdunski et al., 1974). If the reaction of IgG 45-394 with its oligosaccharide haptens would proceed via an elementary binding step, then the rate of association should increase slightly with decreasing chain length. The disaccharide probably has less steric restriction in the association step, and it may have more possibilities to bind than the larger oligomers. In most kinetic studies reported so far for the binding of small hapten molecules to antibodies, very high association rate constants of the order of 10^8 M^{-1} s^{-1} were reported (Froese and Sehon, 1975). They were interpreted as being nearly diffusion controlled. Sometimes it was argued that the limit for the diffusion-controlled reaction would be even higher and that the reaction with IgG takes place in two steps in which the first diffusion-controlled step was not resolved (Haselkorn et al., 1974). The association rate constants found in the present study for the binding of oligosaccharides are by two to four orders of magnitude slower than expected for diffusion-controlled reactions. The association rate constant for the binding of the disaccharide was only about 10^4 M^{-1} s^{-1} which is unexpectedly small for any simple bimolecular binding reaction of this type.

A mechanism consistent with the experimental results and the above considerations must include: (i) a ligand independent rate-determining step for dissociation; (ii) an apparent rate constant of association which increases with increasing chain length in spite of the rate constant of the first binding step being chain-length independent and probably diffusion controlled; (iii) a single observable relaxation time. The simplest mechanism fulfilling these requirements consists of a rapid preequilibrium of reactants with a precomplex (AH), followed by the formation of a final complex AH via a slow isomerization step:

$$A + H \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (AH) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} AH \text{ with } k_{21} \gg k_{23} \qquad (2)$$

Similar mechanisms have been suggested for other systems (Luthy et al., 1973; Quast et al., 1974; Riesner et al., 1976; Bagshaw et al., 1974). The experimental rate constant of dissociation k_d equals k_{32}. This isomerization may reflect a conformational change in the liganded IgG molecule and may not depend on the size and chemical nature of the hapten. This step could be possibly connected with the ligand-induced local conformational changes detected in this system by spectroscopic methods (Jaton et al., 1975a,b). The possible molecular events induced by ligand binding have been suggested to consist of a rearrangement of IgG domains on the basis of recent x-ray studies (Huber et al., 1976). The independence of k_d from hapten is supported by the kinetic data on the binding of aromatic haptens to mouse immunoglobulin MOPC 460 (Huber et al., 1976; Pecht, 1976). From this study an isomerization constant for the liganded immunoglobulin of 13 s^{-1} was derived; this value agrees with our k_d value of 12–16 s^{-1}. The independence of the activation energy of dissociation from the chain length of the oligosaccharides again suggests that the rate of the dissociation process is determined by the same elementary step, e.g., the isomerization of the liganded antibody.

According to mechanism 2, the experimental association rate constant k_a is the product of the preequilibrium binding constant K_1 and the rate constant k_{23}. If the same isomerization is triggered by all oligosaccharides, the variation of k_a would be attributed to decreasing values of K_1 with decreasing size of the oligosaccharides. For the preequilibrium the usual kinetic behavior for elementary binding steps with the rate constant of association k_{12} of the order of 10^8 M^{-1} s^{-1} (Froese and Sehon, 1975; Haselkorn et al., 1974) and the dissociation rate constant decreasing with increasing K_1 is expected.

A small equilibrium constant $K_1 \ll (\bar{c}_H + \bar{c}_A)^{-1}$ explains the observation of a single relaxation time. For $K_1 < 1000$ M^{-1} the preequilibrium is on the side of the free reactants even for the highest concentrations used, $\bar{c}_H + \bar{c}_A = 10^{-4}$ M. The fast relaxation phase corresponding to the first step in eq 2 will then have an extremely small amplitude and may escape detection. No indication for a leveling-off of the linear dependence of τ^{-1} on $\bar{c}_A + \bar{c}_H$ was detectable up to concentrations of 10^{-4} M. This is another indication for $K_1 < 1000$ M^{-1}. Measurements at concentrations larger than 10^{-4} M were hampered by the small amplitude of the relaxation process.

In the framework of mechanism 2, the activation energy which was determined for k_a is an apparent activation energy $E_a = \Delta H_1 + E_{23}$, where ΔH_1 is the enthalpy change association with the preequilibrium and E_{23} is a true activation energy which corresponds to k_{23}. A negative value of E_a which may arise when ΔH_1 is sufficiently negative would provide direct evidence for the presence of a fast preequilibrium (Pörschke and Eigen, 1971). For our system E_a is clearly positive for all chain length which, however, does not outrule mechanism 2 since neither the sign nor the magnitude of ΔH are known. The experimental data for E_a at different chain lengths are not sufficiently accurate for the detection of a possible small chain-length dependence of ΔH_1 and E_a.

Recently two coupled relaxation steps were observed for the DNP-binding myeloma protein, MOPC 460 (Pecht, 1976; Lancent and Pecht, 1976). A mechanism was proposed in which the hapten binds with different affinities to two conformers of the immunoglobulin. The binding constants of the hapten to the two conformers differed only by a factor of 10. Mechanism 2 is a special case of this more general mechanism, if (i) the affinities of the oligosaccharides for the two conformers differ by a much larger factor, and (ii) the isomerization equilibrium for the unliganded conformer of IgG 45-394 is shifted very much to the low affinity form which slowly converts to the high affinity form. Oligosaccharides might bind nearly exclusively to one conformer of their specific antibody in contrast to the binding of DNP-hapten to the myeloma protein MOPC 460.

Kinetic data on the Fab fragment of IgG 45-394 are incomplete but the results obtained with the hexasaccharide at one temperature do not significantly differ from those obtained with IgG. This is most easily explained by assuming that the isomerization step also takes place in the Fab fragment.

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