Binding Studies on Anti-Fructofuranan Mouse Myeloma Immunoglobulins A47N, A4, U61, and E109†

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ABSTRACT: Four murine myeloma immunoglobulins, A4, A47N, U61, and E109, have been studied for their binding affinities with inulin and a series of oligosaccharides derived from inulin. The results indicate that the combining site of these immunoglobulins shows highest complementarity for a trifructofuranosyl sequence (A4 and A47N) and a tetrafructofuranosyl sequence (U61 and E109). The size of the combining area of the immunoglobulin E109 derived from the antigenic determinant (approximately 15 × 14 × 10 Å) agrees well with the size observed on a hypothetical space model of the Fv portion of E109 (Potter, M., Rudikoff, S., Padlan, E. A., and Vrana, M. (1976), Antibodies in Human Diagnosis and Therapy, Haber, E., and Krause, R. M., Ed., New York, N.Y., Raven Press).

This laboratory is interested (Vrana et al., 1976) in murine myeloma immunoglobulins with anti-fructofuranan specificity. Firstly, to study the combining site size by measurement of ligand–antibody interaction (Jolley and Glaudemans, 1974) and, secondly, in order to elucidate the supposed difference between anti-(2 → 1)- and anti-(2 → 6)-fructofuranans.

There have been reports on binding studies between fructose-containing ligands and anti-fructofuranans (Grey et al., 1971; Lundblad et al., 1972; Cisar et al., 1975). In the latter work the binding of two murine IgA's[1] with anti-inulin specificity (W3082 and U61) was studied with a number of (2 → 1)-linked Fru*f* oligosaccharides which were all terminated by a (2 → 6)-linked glucose unit. In inulin, the polysaccharide chains are nonreducing and are terminated by an α-D-Glc*p* unit linked as in sucrose. Thus, the ligands studied by Cisar et al. have terminal glucose linkages, unlike those encountered in the (supposedly) homologous antigen.

Using the method of ligand-altered fluorescence of immunoglobulins (Pollet and Edelhoch, 1973; Jolley et al., 1973; Jolley and Glaudemans, 1974), we have studied the binding of oligosaccharides containing (2 → 1)-linked β-D-Fru*f* residues terminated by a (2 → 1)-linked α-D-Glc*p* moiety (thus corresponding to fragments of inulin) with mouse myeloma immunoglobulins A4, A47N, U61, and E109. Inulin itself was included in the study.

The heavy chains of these immunoglobulins have been analyzed for their amino acid sequence (M. Vrana, personal communication). All four immunoglobulins show the presence of tryptophan in, and near, the first hypervariable (hv) region of the heavy chain (H33 and H37), as well as tyrosine in the first and second hv region of the heavy chain (H32 and H58). These residues may be involved in the ligand-induced altered fluorescence we have observed for these four proteins.

Materials and Methods

Immunoglobulins. All plasmacytomas (ABE4, ABE47N, UPC61, and EPC109) were of BALB/c origin (Lieberman et al., 1975) and ascites were generously supplied by Dr. M. Potter (NCI). The proteins (IgAκ) were purified from ascites by Dr. M. Vrana, as described earlier (Vrana et al., 1976) and obtained in the monomeric form. Pepsin fragments (Fab') were prepared as described by Inbar et al. (1971) and Rudikoff et al. (1972).

Carbohydrates. Inulin (Pfanstiehl) was used without further purification. Methyl α-D-Glc*p* (Pfanstiehl) and methyl β-D-Fru*f* (gift from Dr. R. K. Ness) were washed (in aqueous solution) through a small charcoal column in order to remove a small amount of fluorescent impurity. Sucrose (Baker) was dialyzed and recrystallized to remove polymeric impurities (Hehre and Sugg, 1942).

The oligosaccharides, 1-kestose ($F_2G^1{}_1$), nystose ($F_3G^1{}_1$), and the corresponding pentasaccharide, $F_4G^1{}_1$, were prepared by the action of a crude fraction of α-amylase (Sigma Chemicals) from *Aspergillus oryzae* on sucrose,[2] basically according to the procedure of Pazur (1962). Fractionation of 800 mg of the crude, lyophilized reaction mixture by paper chromatography (Whatman 3MM, ethyl acetate–pyridine–water, 10:4:3, 24 h) afforded pure 111 mg of 1-kestose ($F_2G^1{}_1$), 97 mg of nystose ($F_3G^1{}_1$), 7.6 mg of $F_4G^1{}_1$, and traces of $F_5G^1{}_1$.

The identity of the fructosylsucroses was established in the following way. (a) The specific optical rotation for $F_2G^1{}_1$ was found to be +26.8° (*c* 0.9, water) and for $F_3G^1{}_1$ +8.6° (*c* 0.8, water); the literature values are +28° (*c* 2, water) (Pazur, 1962) and +10.0° (*c* 0.36, water) (Kamerling et al., 1972a). (b) Total acid hydrolysis and quantitative paper chromatography of $F_2G^1{}_1$ and $F_3G^1{}_1$ gave the following ratios of fructose to glucose: 2.0:1.0 and 2.7:1.0, respectively. (c) ^{1}H NMR

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[1] Abbreviations used are: Glc*p*, glucopyranos(yl)ide; Fru*f*, fructofuranos(yl)ide; $F_1G^1{}_1$, β-D-Fru*f*-(2 → 1)-α-D-Glc*p* (sucrose); $F_2G^1{}_1$, β-D-Fru*f*-(2 → 1)-β-D-Fru*f*-(2 → 1)-α-D-Glc*p* (1-kestose); $F_3G^1{}_1$, the corresponding tetrasaccharide (nystose); $F_4G^1{}_1$, the corresponding pentasaccharide; $F_nG^1{}_1$, [(→1)-β-D-Fru*f*-(2→)$_n$1)-α-D-Glc*p* (inulin); $F_1G^6{}_1$, β-D-Fru*f*-(2 → 6)-D-Glc; $F_2G^6{}_1$, β-D-Fru*f*-(2 → 1)-β-D-Fru*f*-(2 → 6)-D-Glc; $F_3G^6{}_1$, the corresponding tetrasaccharide (thus, the superscripts 1 and 6 for G are to distinguish between the series of oligosaccharides with (2 → 1)-linked and (2 → 6)-linked glucose terminal units); IgA, immunoglobulin A; PBS, 0.05 M phosphate-buffered saline (0.85%); Tris-HCl, 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4).

[2] In the reference by Pazur (1962), "Takadiastase" is recommended as a source of transfructosylase. In the Sigma Catalogue (April 1976, page 390), Takadiastase is reported to be "the trademark for an *Aspergillus oryzae* diastase (α-amylase)". We therefore employed such an α-amylase preparation in the hope that it would contain the desired transfructosylase. Subsequent isolation and characterization of the oligosaccharides obviously confirmed this.

TABLE I: Maximum, Ligand-Induced Changes in Fluorescence (in %) of the Myeloma Proteins E109, A47N, A4, and U61, Monitored at 340 nm after Excitation at Either 295 or 280 nm.

	E109				A47N				A4				U61			
	Monomer		Fab′		Monomer		Fab′		Monomer		Fab′		Monomer		Fab′	
Ligands	295	280	295	280	295	280	295	280	295	280	295	280	295	280	295	280
Me α-D-Glc*p*	−2.7		−0.8	−0.7	−2.1	−0.6	−1.2	−1.2	−2.4	−0.3	−1.1	−1.0	−1.2	~0	−0.9	−0.6
Me β-D-Fru*f*	−1.8	−2.0	−0.6	−2.1	−4.7	−4.3	−3.2	−5.2	−1.8	−2.2	−2.6	−6.3	−0.5	<−1	−0.4	−2.0
Sucrose ($F_1G^1_1$)	−7.9				−4.2				−7.5				−4.5	−7.2		−7.4
1-Kestose ($F_2G^1_1$)	−3.4			−6.5	−4.5			−6.1	−6.2				−1.4	−2.7		
Nystose ($F_3G^1_1$)	−2.1	−6.5		−7.3	−2.7	−6.5		−6.6	−2.6	−6.0	−1.9	−7.7	<−1	−2.5		−2.8
$F_4G^1_1$	~0	−6.6		−6.7	<1	−4.8		−4.3		−7.5		−6.3		−3.9		−2.8
Inulin ($F_nG^1_1$)	−3.7		−4.1				−5.7				−4.9		<−2	−9.1		−5.2

spectra were run at 220 MHz and 70 °C using D_2O-exchanged samples of $F_2G^1_1$ and $F_3G^1_1$, as well as sucrose ($F_1G^1_1$) and inulin ($F_nG^1_1$). Because of the low solubility of inulin in water, Me_2SO-d_6 was used as solvent. Partial interpretation of the spectra was possible, especially after comparison with ^1H NMR data on sucrose (de Bruyn et al., 1975) and derivatives of fructosyl oligosaccharides (Binkley et al., 1969; Streefkerk et al., 1974). For the glucose units of all four compounds, the protons H-1, H-2, and H-4 can be readily assigned and occur within the following narrow ranges: δ 5.21–5.26, 3.20–3.22, and 3.13–3.15 ppm, respectively. Also, the coupling constants $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ vary little: 3.7, 9.1–9.5, 9.1–9.3, and 8.8–9.2 Hz, respectively. As far as the Fru*f* residues are concerned, all protons (except for H-3 and H-4) occur within a very narrow range (δ 3.40–3.75 ppm) so that no first-order analysis is possible here. In the Fru*f* units of all four compounds the chemical shift of H-4 is remarkably constant (δ 3.82–3.84 ppm). This also holds true for H-3 (δ 3.95–3.99 ppm) in $F_1G^1_1$, $F_2G^1_1$, and $F_3G^1_1$; for inulin a slightly larger range is observed: one H-3 resonates at δ 3.97, one at δ 4.00, and the remaining ones at δ 4.06 ppm. The coupling constants $J_{3,4}$ and $J_{4,5}$ vary little throughout the series of compounds: 7.6–8.0 and 6.4–7.0 Hz, respectively. Therefore, the ^1H NMR data support the conclusion that all four compounds studied belong to the same homologous series of inulin-related oligosaccharides. (d) Mass spectra of pertrimethylsilyl (Me_3Si) derivatives (Kamerling et al., 1972b) of the oligosaccharides were run at 70 eV on a LKB-9000 GC–MS spectrometer at different temperatures: 130 °C for Me_3Si-$F_2G^1_1$ and 160 °C for Me_3Si-$F_3G^1_1$ and Me_3Si-$F_4G^1_1$. Kamerling et al. (1972b) found that Me_3Si-$F_2G^1_1$ and Me_3Si-$F_3G^1_1$ are characterized by a peak at *m/e* 811, while other important peaks are at *m/e* 829, 813, 739, 671, 649, 451, 437, and 361. For the intensities of the peaks the following was found: $I_{451} > I_{437}$ and $I_{811} > I_{813}$ (both compounds), furthermore, $I_{671} > I_{649}$ (Me_3Si-$F_2G^1_1$) and $I_{671} < I_{649}$ (Me_3Si-$F_3G^1_1$). Our spectral data for both compounds agree fully with these results. No literature data are available for Me_3Si-$F_4G^1_1$, but the same important peaks and similar peak intensities were observed for this compound as for Me_3Si-$F_3G^1_1$ (except $I_{811} \approx I_{813}$), supporting the conclusion that $F_4G^1_1$ is the higher homologue of $F_3G^1_1$. (e) The rates of movement on paper (Whatman no. 1, ethyl acetate–pyridine–water, 10:4:3, 16 h) of $F_2G^1_1$, $F_3G^1_1$, $F_4G^1_1$, and $F_5G^1_1$ relative to sucrose were 0.61, 0.41, 0.28, and 0.17, respectively. A plot of these values against the log molecular weight values of these oligosaccharides gave a straight line, confirming their structural relationship (Kowkabany, 1954).

Double-Diffusion Inhibition Experiments. Agar plates were prepared according to Potter et al. (1972). Wells were prefilled with inhibitor solution (0.4 M methyl α-D-Glc*p*, 0.4 M methyl β-D-Fru*f*, 0.2 M sucrose in phosphate-buffered saline). After the inhibitor diffusion was complete, the three purified myeloma proteins U61, A4, and A47N (E109 was excluded because it did not precipitate with inulin) were added to the outer wells and the plates were left at 37 °C. Inulin (0.1%) was added 1.5 h later and the plates were left at room temperature. Precipitin lines were recorded after 45 min, 1 h, and 3 h.

Fluorescence Titrations. Maximum changes in fluorescence (ΔF_{max}) induced by hapten, as well as binding constants (K_a), were determined by fluorescence titration at 25 °C of the immunoglobulins (0.05 OD in Tris-HCl buffer) as described before (Jolley et al., 1973; Jolley and Glaudemans, 1974). Excitation wavelengths of either 280 or 295 nm were used, the fluorescence changes being monitored at 340 nm. For the mono- and disaccharide ligands, the ΔF_{max} values were obtained by adding solid ligand to the protein solution until no further change in fluorescence was observed. In the case of the tri- and higher oligosaccharides, the protein solution was titrated with a solution of the ligand in Tris-HCl buffer and ΔF was recorded after each addition. Extrapolation of a plot of $\Delta F/c$ vs. ΔF to $\Delta F/c = 0$ then gave the ΔF_{max} value, where c is the concentration of the free ligand. For weakly binding ligands having binding constants, K_a, of $\sim 10^4$ M^{-1} or less, the free-ligand concentration exceeds the protein concentration some hundred times or more, in which case $c_{\text{free ligand}}$ is essentially equal to $c_{\text{ligand added}}$. However, for strongly binding ligands this assumption holds true only near the end of the titration. Therefore, in the latter cases, extrapolation of the last part of the plot of $\Delta F/c$ vs. ΔF was used to obtain ΔF_{max}.

The binding constants (K_a) can be determined from the slope of a Scatchard plot of $\bar{\nu}/c$ vs. $\bar{\nu}$ where $\bar{\nu} = \Delta F/\Delta F_{max}$ and c is either $c_{\text{free ligand}}$ or $c_{\text{ligand added}}$ (see above). The free ligand concentration can be computed as follows: $c_{\text{free ligand}} = c_{\text{ligand added}} - \bar{\nu}$[sites], where [sites] is the concentration of binding sites of the protein. Taking the molecular weight of an IgA monomer as 155 000 and of an IgA (Fab′) fragment as 50 000, the [sites] values for an 0.05 OD solution of an IgA monomer and an IgA (Fab′) are 4.6×10^{-7} and 7.2×10^{-7}, respectively. K_a values were obtained by the method of least squares.

Results

Fluorescence Titrations: ΔF_{max} Values. Table I lists the maximum changes (decreases) in fluorescence (ΔF_{max}) for the monomeric myeloma IgA proteins A4, A47N, E109, and U61 as well as for their Fab′ fragments, induced by the following ligands: methyl α-D-Glc*p*, methyl β-D-Fru*f*, sucrose ($F_1G^1_1$), 1-kestose ($F_2G^1_1$), nystose ($F_3G^1_1$), and the pentasaccharide $F_4G^1_1$. The ΔF_{max} for the four Fab′ fragments in the presence of excess inulin is also given.

TABLE II: Association Constants, K_a (M^{-1}), for Various Ligands with the Myeloma Proteins E109, A47N, A4, and U61 Determined by Fluorescence Titrations.[a]

Ligands	E109		A47N		A4		U61	
	Monomer	Fab′	Monomer	Fab′	Monomer	Fab′	Monomer	Fab′
Me α-D-Glc*p*	3.3 (± 0.2) × 10^2	*b*	1.5 (±0.2) × 10^2	*b*	1.4 (±0.2) × 10^2	*b*	*b*	*b*
Me β-D-Fru*f*	*b*	*b*	1.7 (±0.1) × 10^2	1.35 (±0.02) × 10^2	*b*	2.03 (±0.06) × 10^2	*b*	*b*
Sucrose ($F_1G^1{}_1$)	0.93 (±0.07) × 10^3	*c*	1.20 (±0.03) × 10^3	*c*	1.05 (±0.05) × 10^3	*c*	0.68 (±0.07) × 10^3	0.53 (±0.05) × 10^3
1-Kestose ($F_2G^1{}_1$)	0.97 (±0.05) × 10^4	1.2 (±0.1) × 10^4	0.71 (±0.06) × 10^4	1.04 (±0.04) × 10^4	1.10 (±0.05) × 10^4	*c*	1.32 (±0.07) × 10^4	*c*
Nystose ($F_3G^1{}_1$)	1.7 (±0.1) × 10^5	2.3 (±0.1) × 10^5	1.09 (±0.02) × 10^5	1.15 (±0.02) × 10^5	2.18 (±0.03) × 10^5	1.9 (±0.2) × 10^5	1.34 (±0.04) × 10^5	1.5 (±0.1) × 10^5
$F_4G^1{}_1$	3.6 (±0.2) × 10^5	4.0 (±0.2) × 10^5	1.1 (±0.1) × 10^5	1.3 (±0.1) × 10^5	3.8 (±0.2) × 10^5	1.7 (±0.1) × 10^5	3.1 (±0.1) × 10^5	2.7 (±0.2) × 10^5
Inulin ($F_nG^1{}_1$)	11 (±1) × 10^5	6.6 (±0.6) × 10^5	*c*	2.2 (±0.2) × 10^5	*c*	4.2 (±0.3) × 10^5	*c*	3.1 (±0.2) × 10^5

[a] All titrations were done at that excitation wavelength giving the highest ΔF_{max} (Table I). [b] K_a could not be determined, ΔF_{max} too low. [c] K_a not determined.

Ouchterlony double-diffusion experiments showed that of the four monomeric immunoglobulins, only E109 failed to precipitate with inulin. Measurement of the ΔF_{max} values (and also the association constant) for any monomeric protein with inulin would obviously be fruitless if this is a precipitating system. A measurement of ΔF as a function of inulin added to an immunoglobulin is only meaningful in the case of E109. Indeed, the ΔF_{max} for this system (see Table I) is only slightly lower than for the system E109 (Fab′)-inulin.

Association Constants. Table II lists the association constants, K_a, for the four monomeric immunoglobulins and for their Fab′ fragments with the various ligands. When the absolute value of ΔF_{max} is around 2% or less, no accurate K_a value may be found. It can be inferred from Table I that a very low ΔF_{max} at one excitation wavelength does not necessarily mean that the ligand is not binding to the protein, as excitation at another wavelength sometimes gave a much higher ΔF_{max}, and therefore a measurable K_a. See, for instance, the system E109 monomer–nystose in Table I.

Double-Diffusion Experiments. Monomeric immunoglobulins A4, A47N, E109, and U61 were run on agar vs. 0.1% inulin. A4, A47N, and U61 showed a single precipitin line, but E109 did not precipitate. Methyl β-D-Fru*f* (0.4 M), methyl α-D-Glc*p* (0.4 M), and sucrose (0.2 M) were tested for their power to inhibit the three precipitating systems. Only methyl α-D-Glc*p* failed to inhibit precipitation.

Discussion

Inulin is a linear polysaccharide containing β-D-Fru*f* units linked 2 → 1, and terminated, as in sucrose, by a similarly linked α-D-Glc*p* residue (Drew and Haworth, 1928; Haworth and Learner, 1928; Hirst et al., 1950; Feingold and Avigad, 1956; Holzer et al., 1957).

Methylation studies have shown that the number average molecular weight of inulin is approximately 5000, involving about 28 intercatenary Fru*f* residues, one nonreducing terminal Fru*f* residue, and at the other terminal end a nonreducing Glc*p* residue (Hirst et al., 1950; Boggs and Smith, 1956). Due to the mode of isolation of the tetramethylhexoses in the above studies of inulin, the value of ~5000 for the molecular weight must be an upper limit. This, because any loss of the more volatile, highly methylated terminal sugar residues, would be reflected in a higher apparent molecular weight.

We have assumed that *one* molecule of inulin binds to only *one* combining site of the immunoglobulins under the conditions of our titrations. From the work of Segal et al. (1974) on the structure of immunoglobulin Mc603 we know that the N-terminal portion of a Fab′ fragment has a width of approximately 40 Å. It would thus appear possible that two immunoglobulin combining sites could bind to inulin, since inulin in its extended form has a length of approximately 100 Å. However, in the case of the myeloma proteins studied here it can be seen in Table II that the association constants of $F_4G^1{}_1$ with the anti-inulin Fab′ fragments are essentially identical to those of inulin with the same fragments; the latter constants were calculated using the above-mentioned assumption. Secondly, in the case of E109 and inulin, no precipitation occurred upon double diffusion. Thus, that preparation of E109 monomer is definitely incapable of binding more than one immunoglobulin combining site per molecule of inulin, otherwise cross-linking would occur, and precipitation takes place. In the case of the other three immunoglobulins, the observed precipitation with inulin must therefore be attributed to noncovalent recombination of monomers to polymers in the purified immunoglobulin solution; it has been reported that this can occur with IgA's (Mushinski, 1971; Heremans, 1974). Above all, however, under the conditions of the titration, the antigen excess at site saturation is considerable (10–30 times) and it is, therefore, unlikely that more than one antibody combining site binds per molecule of inulin.

The apparent failure of E109 monomer to spontaneously recombine to dimers or polymers gave us the opportunity to accurately measure the affinity constant of an antigen and its *whole* immunoglobulin. Although there have been previous reports of affinity measurements between antigens and antibodies (Stone and Metzger, 1968; Noble et al., 1969), to our knowledge this is the first such recorded instance using the fluorescence titration method. It can be seen in Table II that the constants of association obtained for inulin with E109 monomer and the corresponding Fab′ fragment are very similar, albeit not identical. This similarity becomes even more apparent when evaluating the free energy of binding for both systems, which can be calculated from the K_a by the equation $\Delta G = -RT \ln K_a$: −8.0 kcal for E109 (Fab′)-inulin, and −8.3 kcal for E109 (monomer)-inulin. It thus appears that for this particular system, the titration of immunoglobulin with macromolecular antigen is valid.

It can also be seen in Table II that methyl α-D-Glc*p* ap-

TABLE III: Percentage Contributions[a] of the Fructofuranosyl Moieties to the Binding Energies of 1-Kestose, Nystose, the Pentasaccharide F4G1, and Inulin with the Myeloma Proteins E109, A47N, A4, and U61.[b]

Ig	Ligand		Fructofuranosyl moiety no.				
			1[c]	2	3	4	5 → *n*
E109	1-Kestose	$F_2G^1{}_1$	73.9	26.1			
	Nystose	$F_3G^1{}_1$	56.1	19.7	24.2		
		$F_4G^1{}_1$	53.2	18.8	22.9	5.1	
	Inulin	$F_nG^1{}_1$	51.0	18.0	22.0	4.9	4.1
A47N	1-Kestose	$F_2G^1{}_1$	78.3	21.7			
	Nystose	$F_3G^1{}_1$	61.0	16.9	22.1		
		$F_4G^1{}_1$	60.6	16.8	22.0	0.6	
	Inulin	$F_nG^1{}_1$	57.6	16.1	20.9	0.5	4.9
A4	1-Kestose	$F_2G^1{}_1$	74.8	25.2			
	Nystose	$F_3G^1{}_1$	57.0	19.2	23.8		
		$F_4G^1{}_1$	55.9	18.9	23.4	1.8	
	Inulin	$F_nG^1{}_1$	53.7	18.2	22.5	1.7	3.9
U61	1-Kestose	$F_2G^1{}_1$	67.4	32.6			
	Nystose	$F_3G^1{}_1$	53.9	26.0	20.1		
		$F_4G^1{}_1$	50.9	24.5	19.0	5.6	
	Inulin	$F_nG^1{}_1$	50.6	24.5	18.8	5.6	0.5

[a] These data were obtained by deriving the free energy of binding, ΔG, from the measured K_a. For instance: $\Delta G_{kestose} - \Delta G_{sucrose}$ would give the $\Delta G_{fructose}$ (2) in 1-kestose (expressed in percentages of the total binding energy of 1-kestose). [b] It is assumed that the contribution of the glucose moiety is negligible. [c] Fru*f* moiety located next to the glucose moiety.

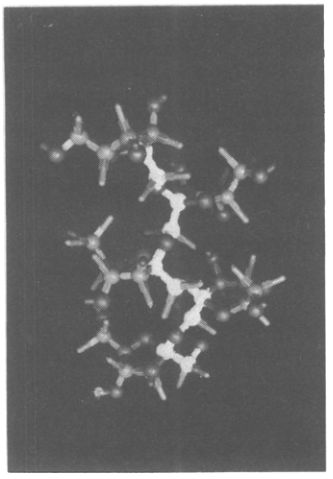

FIGURE 1: Molecular model of a pentamer unit of a chain of (2 → 1)-linked β-D-Fru*f* residues. The backbone of (2 → 1)-linkages is shown in white.

parently binds to the E109, A47N, and A4 monomers. Table I shows that the ligand-induced changes in fluorescence in these cases are marginally low and that the ΔF_{max} values for the corresponding Fab′ fragments with this ligand are too small to allow a binding constant to be determined with confidence. One would expect a Fab′ fragment to register a higher ligand-induced fluorescence change when compared to the monomer, since the background fluorescence of the now removed Fc portion no longer contributes (as is indeed observed for all other ligands; Table I). The data on methyl α-D-Glc*p* may therefore very well mean that this simple glycoside binds to E109, A47N, and A4 at a position other than the immunoglobulin combining site, perhaps somewhere on the Fc portion. Indeed, methyl α-D-Glc*p* was incapable of inhibiting the precipitation of A47N, A4, or U61 with inulin, thereby confirming that binding takes place outside the antibody combining site. In contrast, methyl β-D-Fru*f* seems to bind equally well with A47N monomer or its Fab′ fragment. It is unfortunate that no measurements of this ligand other than with A4

TABLE IV: Comparison of Constants of Association (K_a) of Different Ligands Containing Fructose and Glucose with Myeloma Protein U-61.

Ligand	K_a (M^{-1}) Fluorescence titration	K_a (M^{-1}) Precipitin inhibition	Reference
$F_1G^1{}_1$	6.3×10^2	5.0×10^2	Cisar et al., 1975
	6.8×10^2		This paper
$F_1G^6{}_1$		8.1×10^2	Cisar et al., 1975
$F_2G^6{}_1$	3.6×10^5	2.5×10^5	Cisar et al., 1975
$F_2G^1{}_1$	1.32×10^4		This paper
$F_3G^6{}_1$	3.6×10^5	3.6×10^5	Cisar et al., 1975
$F_3G^1{}_1$	1.34×10^5		This paper
$F_4G^1{}_1$	3.1×10^5		This paper

(Fab′) could be conducted, but the ligand is a good inhibitor of the precipitin reaction between A47N, A4, and U61 with inulin, thus indicating that binding takes place in the combining sites of these immunoglobulins.

The binding constants for sucrose ($F_1G^1{}_1$) to the four immunoglobulins studied show that there, nevertheless, must be a small contribution of the glucose moiety to the binding of $F_1G^1{}_1$, as this ligand binds several times better than methyl β-D-Fru*f* (Table II). Looking at the ligands $F_1G^1{}_1$, $F_2G^1{}_1$, $F_3G^1{}_1$, and $F_4G^1{}_1$ in Table II shows that the binding affinities in this series reach an optimum value for $F_4G^1{}_1$, which is essentially identical with that of inulin with the same Fab′ fragments. From the binding constants listed in Table II, it is possible to compute the fractional binding contribution of each monosaccharide moiety in a ligand to the binding energy of that whole ligand (Table III). It is clear, from the values for the oligosaccharides, that highest complementarity is achieved when four (in the case of E109 and U61) or three (in the case of A47N and A4) Fru*f* residues occupy the binding site. The immunodominant part of the oligosaccharide ligand appears to be the glucose-terminal Fru*f* unit, because methyl α-D-Glc*p* either binds very weakly or not at all (see above).

Figure 1 shows a possible conformation for a chain of 2 → 1 linked D-Fru*f* units, which avoids dipole–dipole interactions

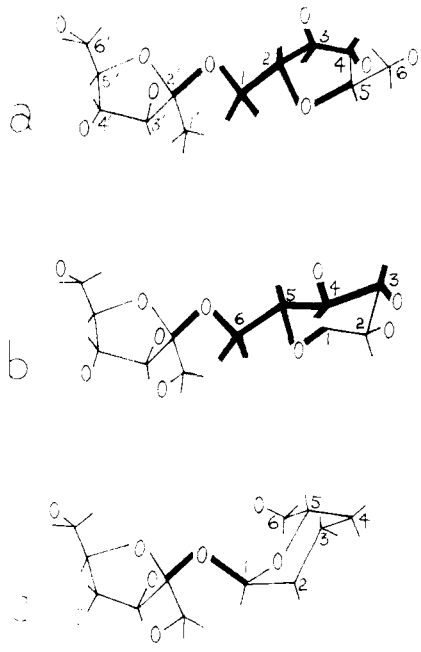

FIGURE 2: Structural relationships between a (2 → 1)-linked β-D-Fru*f* disaccharide (a) and two differently linked D-Fru*f*-D-Glc disaccharides; (b) β-D-Fru*f*-(2 → 6)-D-Glc and (c) β-D-Fru*f*-(2 → 1)-α-D-Glc*p*. The heavy lines indicate structural similarities between these compounds.

for the ring- and intersaccharidic oxygen atoms (exo-anomeric effect; Lemieux and Koto, 1974). Inulin would be a very compact molecule with the intersaccharidic oxygen chain situated centrally. The size of the binding site in these anti-inulin immunoglobulins is therefore thought to be approximately equal to the length of the tri- or tetra-Fru*f* segment in inulin, with maximum dimensions of approximately 14 × 14 × 7 Å (A47N, A4) or 15 × 14 × 10 Å (E109, U61). A hypothetical model (see Potter et al., 1976) of the Fv portion of E109, built by E. Padlan and M. Vrana (NIH), shows a combining site which agrees quite well with these dimensions.

Table III shows that in E109, A47N, and A4 the second Fru*f* residue binds somewhat less strongly than residue number three. If binding is mediated mostly through one side of the immunoglobulin combining site, the above finding can be explained by the staggered nature of the Fru*f* "side groups" attached to the intersaccharidic chain of inulin (Figure 1). This differentiation does not appear to occur in U61.

It is interesting to compare the binding affinities of our ligands and U61 with those published by Cisar et al. (1975) for their ligands and U61. These authors measured affinity constants of ligands ranging from sucrose to oligosaccharides containing (2 → 1)-linked β-D-Fru*f* residues terminated, not by a (2 → 1)-linked glucose unit (as in our case) but by a (2 → 6)-linked D-glucose residue (Table IV). There is excellent agreement between the K_a for sucrose and U61 as found by us, and by Cisar et al. The K_a for the tetrasaccharide $F_3G^1{}_1$ (1.34×10^5 M^{-1}) is close to the K_as found for the trisaccharide $F_2G^6{}_1$ (3.6×10^5 and 2.5×10^5 M^{-1}). The same holds true for the K_as of $F_4G^1{}_1$ and $F_3G^6{}_1$ (3.1×10^5 and 3.6×10^5 M^{-1}, respectively). As pointed out before, it is probable that U61 binds only to the Fru*f* units in our oligosaccharides. However, in the oligosaccharides terminated by a (2 → 6)-linked D-glucose residue, it appears that U61 may bind to a part of the glucose residue. Otherwise, $F_2G^6{}_1$ could not bind with an affinity similar to that found for $F_3G^1{}_1$. Indeed, if one considers the molecular models for the oligosaccharides involved (Figure 2) it appears that a (2 → 6)-linked D-Glc*p* residue is capable of mimicking a substantial part of the (2 → 1)-linked Fru*f* moiety (compare Figure 2a and 2b). Thus, this would readily explain the similarity in binding between $F_2G^6{}_1$ and $F_3G^1{}_1$ with U61, and between $F_3G^6{}_1$ and $F_4G^1{}_1$. It is quite clear that the glucose terminal end of the inulin molecule (Figure 2c) is very unlike the intercatenary poly(Fru*f*) structure. Therefore, these data again confirm for U61 that it is an immunoglobulin which binds inulin terminally at the Fru*f* end and/or along the antigen chain.

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Amino Acid Sequence of Normal (Microheterogeneous) Porcine Immunoglobulin λ Chains†

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ABSTRACT: The partial amino acid sequence of pooled, microheterogeneous pig immunoglobulin λ chains was determined previously (Franěk, F. (1970), *FEBS Lett. 8*, 269; Novotný, J., and Franěk, F. (1975), *FEBS Lett. 58*, 24). In the present study, citraconylated pig λ chains were digested by trypsin under conditions in which some of the ε-amino groups of lysine residues unmask. The resulting fragments were purified by gel filtration and ion-exchange chromatography at pH 3.0 in buffers containing urea; some of the fragments were found to be of intermediate size (i.e., larger than normal tryptic peptides but smaller than "citraconyl" peptides), thus permitting overlap information and amino acid sequences of all the 14 tryptic peptides to be deduced from amino acid compositions and partial amino acid sequences of selected fragments. In addition to completing the major amino acid sequence of pig immunoglobulin λ chains, the present study demonstrates that it is possible to sequence microheterogeneous proteins with a suitable fragmentation strategy.

Sequencing of microheterogeneous polypeptide chains (such as immunoglobulin chains from pooled nonspecific immunoglobulins) involves the difficulty of isolating and sequencing fragments of non-uniform amino acid sequence. Most of the current approaches to this problem are due to pioneering work of Porter and his group on rabbit immunoglobulin γ chains (Porter, 1967, 1973) and Cebra's group on guinea pig immunoglobulin γ chains (Cebra et al., 1971). Previous work in our laboratory on pooled porcine λ chains led to the tentative amino acid sequence of large segments of these chains, namely, residues 1–46 and 72–93 from the V region[1] (Franěk et al., 1969b; Franěk, 1970), and residues 113–168, 169–206, and 207–214 (Novotný and Franěk, 1975a) from the constant (C) region (i.e., the sequence of the constant region was complete except for overlaps between residues 168–169 and 206–207). The two intrachain disulfide bonds were localized to half-cystine residues 21–89 and 136–195, respectively (Franěk et al., 1968). The purpose of the present publication is twofold: (1) To provide amino acid sequence data sufficient for derivation of the complete λ chain sequence; and (2) to report fragmentation and isolation procedures permitting better quantitation and improved yields of chain fragments. The latter point is of considerable importance in sequence work on microheterogeneous proteins; if a fragment is obtained from a homogeneous protein in low yield (e.g., 15% of the theoretical), this fact has minor impact on the elucidation of the complete primary structure of the protein. If, however, a fragment has been obtained from a microheterogeneous protein in 15% yield, it cannot be excluded that the remaining 85% is represented by variants with properties (amino acid sequence, electric charge, solubility) other than those of the fragment obtained. It would be extremely laborious and often impossible to prove unequivocally that the low yield of the fragment is solely due to technical difficulties such as incomplete cleavage and losses during isolation procedures.

The present work shows that it is possible to derive the complete amino acid sequence of major variants of pooled immunoglobulin λ chains by applying a single step fragmentation procedure, namely, partial tryptic hydrolysis.

Materials and Methods

Materials. Preparation of pig λ chain and its resolution into λ(15), λ(16), and λ(17) subpopulations was described previously in detail (Franěk and Zorina, 1967). DCC-trypsin was purchased from Calbiochem. Pepsin, thermolysin, citraconic anhydride, trinitrobenzenesulfonic acid, ethylenimine, and dithiothreitol were also commercial products. The reagents used for automatic Edman degradation were purchased from Beckman. Those used for manual Edman degradation were pure commercial products repurified as recommended by Edman (1970). Urea and guanidine hydrochloride used for preparation of buffers were prepared from technical products by the Service Laboratory of the Institute of Organic Chemistry and Biochemistry (Prague). Optical absorbance of 6 M solutions of these compounds at 280 nm was less than 0.1. The

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[1] Abbreviations used: V region, variable region; C region, constant region; DCC-trypsin, diphenylcarbamoyl chloride treated trypsin; Pth-amino acid, phenylthiohydantoin amino acid; SE-Sephadex, sulfoethyl-Sephadex C-25.