Reversible and irreversible cross-linking of immunoglobulin heavy chains through their carbohydrate residues

Urs HEIMGARTNER,* Branko KOZULIĆ† and Klaus MOSBACH†

Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, 8093 Zürich, Switzerland

After periodate oxidation and incubation with a dihydrazide, cross-linking of the two heavy chains of immunoglobulins G from several species proceeds specifically through their oligosaccharides. We have used malonic acid dihydrazide, adipic acid dihydrazide and dithiodipropionic acid dihydrazide. The last compound is introduced in this work as a cleavable-carbohydrate-specific cross-linker. It was found that in rabbit and human immunoglobulins the degree of cross-linking was strongly dependent on the oxidation conditions but only very weakly dependent on the concentration and size of the dihydrazides. Papain cleavage of the cross-linked rabbit IgG indicated that the cross-linking occurred predominantly, if not exclusively, in the Fc region, probably through the two glycans linked to Asn-297 in the C_H2 domain of each of the two heavy chains. The immunoglobulins from sheep, pig, goat and guinea pig show a comparable cross-linking pattern, indicating that the sugar chains from these immunoglobulins have a spatial structure closely related to that of rabbit and human IgG. When dithiodipropionic acid dihydrazide was used as the cross-linker, the cross-link could be cleaved by mercaptoethanol.

INTRODUCTION

IgG is a glycoprotein containing approx. 3% carbohydrate. The main part of the sugar of IgG occurs in the C_H2 domain and is N-linked to Asn-297 of both heavy chains [1]. These sugar chains are an integral feature of all normal IgG class antibodies. A small amount of additional N-linked sugars has been found in human IgG [2] and in rabbit IgG [3]. These sugars are situated in the Fab part and their presence depends on the availability of an Asn-X-Ser(Thr) sequence in the hypervariable region [4]. O-Linked oligosaccharides, that are species- and allotype-specific, may be attached to the hinge region of IgG [1].

Details on the conformation and spatial structure of the glycans attached to Asn-297 are at present available only for rabbit and human IgG. The information has been gained by n.m.r. [5] and X-ray analysis of rabbit [6] and human [7] Fc fragments. These studies were complicated by heterogeneity of the glycan chains which consist of a multiple set of structures based on four different types of mannosyl-chitobiosyl cores [8]. The crystal data of the Fc fragment indicate that the two sugar chains lie opposite each other across the interstitial region which separates the two C_H2 domains [6]. It has been suggested that direct carbohydrate-carbohydrate interactions exist in rabbit IgG [6] but not in human IgG [9].

We have shown previously that the subunits of some oligomeric glycoproteins could be cross-linked through their sugar chains, indicating that these chains were spatially close to each other [10,11]. However, this hypothesis could not be proven because there were no details, obtained by other methods, on the spatial structure of sugar chains in these oligomannose-type glycoproteins. Here we have investigated the cross-linking of IgG from several species to see whether the cross-linking pattern is in accordance with the suggested spatial structure discussed above. The cross-linking takes place after periodate oxidation of the monosaccharide residues and subsequent incubation with a

dihydrazide. In addition to testing two cross-linkers differing in size, namely malonic acid dihydrazide (MADH) and adipic acid dihydrazide (AADH), we have introduced in this work a cleavable cross-linker, dithiodipropionic acid dihydrazide (DTDPADH). This reagent offers the possibility of reversibly cross-linking glycoproteins through their sugar chains and it also facilitates the estimation of the number of the introduced cross-linker molecules.

EXPERIMENTAL

Materials

Immunoglobulins, AADH and papain were from Sigma, anhydrous hydrazine was from Aldrich, and sodium periodate and all other chemicals were purchased from Fluka. Ammediol (2-amino-2-methylpropane-1,3-diol) was treated with charcoal (Sigma; C 4386) and recrystallized from acetone/water (20:1, v/v). The materials for electrophoresis were from Bio-Rad. Electrophoresis was performed in a Pharmacia electrophoresis apparatus. Protein A-Sepharose CL 4B was from Pharmacia.

MADH. Diethyl malonate (30 mmol) was dissolved in 30 ml of dry methanol in a round-bottom flask. Under vigorous stirring, 60 mmol of anhydrous hydrazine was added dropwise while cooling in an ice bath. The reaction was allowed to continue for 2 h at room temperature. Crystals (needles) were readily formed at 4 °C. Recrystallization from methanol gave 25 mmol of MADH (83% yield). The melting point was 152 °C, lit. 154 °C [12].

DTDPADH. This compound has previously been synthesized [13], but the procedure described below is more convenient and gives a higher yield. Dithiodipropionic acid (0.2 mol) was refluxed for 3 h in 240 ml of 1 M-HCl in anhydrous methanol. The

Abbreviations used: AADH, adipic acid dihydrazide; MADH, malonic acid dihydrazide; DTDPADH, dithiodipropionic acid dihydrazide.

* Present address: ELCHROM AG, Höschgasse 70, 8008 Zürich, Switzerland.

† Present address: Department of Pure and Applied Biochemistry, Chemical Center, University of Lund, POB 124, SD-221100 Lund, Sweden.

‡ To whom correspondence should be addressed

methanol was evaporated and the remaining dithiodipropionic acid methyl ester was purified by extraction with diethyl ether/1 M-sodium bicarbonate (1:1, v/v). To the dried ester (174.6 mmol; 83 % yield), 160 ml of anhydrous methanol was added and then, after cooling to 0 °C, 355 mmol of anhydrous hydrazine was added. Recrystallization from methanol gave 37 g (155 mmol) of DTDPADH (89 % yield), melting point 125.5 °C, lit. 126 °C [13].

Oxidation of the carbohydrate part of IgG with sodium periodate

In a typical experiment, 2 mg of IgG was dissolved in 0.6 ml of 0.1 M-sodium acetate, pH 4.5, containing 0.1 M-NaCl (acetate buffer) and passed over a Sephadex G-25 column (PD-10; Pharmacia) in order to remove the low-molecular-mass impurities. The IgG fraction was cooled to 0 °C and NaIO₄ (100 mM, freshly prepared) was added to give the desired final concentration. After incubation in the dark, the sample was applied to a PD-10 column to remove excess periodate. The resulting oxidized IgG fraction had a concentration of around 1 mg/ml (recovery 80–90 %).

Cross-linking of oxidized IgG with dihydrazides

The dihydrazide, 0.5 M for MADH and AADH or 0.2 M for DTDPADH (freshly prepared), was dissolved in the acetate buffer and then added to the oxidized IgG to give the desired final concentration (40 mM, if not otherwise specified). The sample was allowed to react for 4 h at room temperature or overnight at 4 °C. The reaction was stopped by passing the sample over a PD-10 column, followed by dialysis against the buffer required for the next step.

Papain cleavage of the cross-linked IgG

Proteolysis of IgG with papain and affinity chromatography over Protein A-Sepharose were done according to [14].

Electrophoresis under denaturing conditions

SDS/PAGE was performed using the discontinuous buffer system of Laemmli [15] or of Bury (ammediol/glycine) [16] with a 5–20 % linear polyacrylamide gradient. The samples were dialysed against the corresponding stacking gel buffer, mixed with sample buffer (containing 2 % SDS and 3 % mercaptoethanol) and either boiled for 4 min at 100 °C (standard proteins) or heated for 45 min at 56 °C (IgG and the cross-linked species). The proteins were visualized by silver staining [17]. Pharmacia low-molecular-mass (designated as *s*₁) and Bio-Rad high-molecular-mass (*s*₂) markers were used. They include (*s*₁): phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa); (*s*₂): myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

Non-denaturing electrophoresis in a polyacrylamide gradient gel

Linear polyacrylamide gradient gels (3–30 %; 2.7 mm thick, 8 cm wide and 8 cm high) were prepared according to the instructions of Pharmacia Fine Chemicals. Electrophoresis was run for 14 h at constant voltage (130 V) in 0.1 M/HCl buffer, pH 9.0. The sample (20–40 μ g per lane) was dialysed against the same buffer. Pharmacia high-molecular-mass markers (*s*) were used: thyroglobulin (669 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (67 kDa).

Electroelution of the cross-linked IgG

After electrophoresis, one part of the gradient gel was quickly stained for proteins [18] in order to facilitate the correct cutting of the band of interest. In the part of the gel which was not stained, a hole was punched out with a cork borer of 5 mm diameter at the position of the IgG monomer. The gel disc was subsequently forced into a glass tube of 5 mm inner diameter, in such a way that a space of approx. 100 μ l was left between the gel and the end of the tube. A dialysis membrane was fixed at the bottom end of the glass tube with silicon tubing and the IgG was electroeluted in 0.1 M-Tris/HCl buffer, pH 9.0. The electroelution was done at constant amperage (2 mA/tube) for 2–4 h. The eluted sample was removed and analysed further by SDS/PAGE. The remaining part of the native gel was stained with Coomassie Brilliant Blue to verify the correct removal of the protein band.

Estimation of the number of DTDPADH molecules introduced into oxidized glycoproteins

This was done by measuring the number of disulphide bonds, as described in [19], in the native glycoproteins and in the glycoproteins after cross-linking with DTDPADH. The protein concentration was determined spectrophotometrically at 280 nm, with the molar absorption data taken from [20].

RESULTS

Rabbit IgG served as a model immunoglobulin since detailed information on its protein and glycan structure is available [3,6,8]. The first experiments were done to check the specificity of the cross-linking reaction and to examine whether the cross-linking is intramolecular or intermolecular. SDS/PAGE analysis

Fig. 1. SDS/PAGE of native, oxidized and cross-linked rabbit IgG under reducing and non-reducing conditions

(a) Samples treated with mercaptoethanol; (b) non-reduced samples. IgG incubated with 100 mM-AADH for 4 h at room temperature is shown in lanes 1. IgG dissociates into its heavy chain (H) and light chain (L) under reducing conditions (a). The oxidized IgG (40 mM-NaIO₄, 0 °C, 2 h) was applied to the gel without incubation with the cross-linker AADH (lanes 2) or after incubation with 0.2 mM-AADH (which gives an approx. 1:1 molar ratio between the cross-linker and the monosaccharide residues of IgG at 1 mg/ml protein concentration) (lanes 3), 4 mM-AADH (lanes 4) or 100 mM-AADH (lanes 5). 2H denotes the proposed heavy chain dimer (a). *s*₁ and *s*₂ shown molecular mass standards, as specified in the Experimental section. The protein load was 0.5 μ g of protein per lane. The buffer system according to Bury [16] was used with a 5–20 % polyacrylamide gradient in the separating gel. All samples were incubated for 45 min at 56 °C, and in (a) additionally with 3 % 2-mercaptoethanol.

of rabbit IgG under reducing conditions reveals that incubation of non-oxidized IgG with AADH (Fig. 1*a*, lane 1), or oxidation with NaIO₄ alone (lane 2) does not alter the protein band pattern of the IgG. However, incubation of the oxidized IgG with AADH produces a new, high-molecular-mass, band with an apparent molecular mass of about 120 kDa (lanes 3–5). This is close to the calculated value of the molecular mass for a heavy chain dimer (108 kDa). A change in the AADH concentration from 0.2 to 100 mM had no influence on the intensity of this upper band (lanes 3–5). The intensity of the band was also constant in an experiment where the IgG concentration was changed from 0.1 to 2 mg/ml (results not shown). Since it had been shown previously that intermolecular cross-linking of glycoproteins was strongly dependent on protein and AADH concentrations [11], these results pointed to intramolecular cross-linking. This hypothesis is supported by results shown in Fig. 1*b*, because SDS-denatured but non-reduced cross-linked IgG migrated in a similar way to untreated IgG (the diffuse band of about 150 kDa). The weak higher-molecular-mass band probably represents two IgG molecules linked together. Since the intensity of that band practically did not change with cross-linker concentration (Fig. 1*b*, lanes 3–5), we believe that this product was formed by intramolecular cross-linking of the IgG dimer present in the preparation (see below), rather than by intermolecular cross-linking of two IgG molecules.

In order to get direct evidence for intramolecular cross-linking, we have electrophoresed the cross-linked IgG in a non-denaturing gradient gel and analysed the protein band that migrated as the untreated IgG monomer (Fig. 2). Compared with the untreated IgG (lane 1), the cross-linking did not change the intensity of the existing bands, nor did it produce a new band in the native gradient gel (lane 2). It should be noted that the native IgG, as well as the dimer that is normally present in IgG preparations [21], showed apparent molecular masses that were higher than expected, as reported previously [22]. SDS/PAGE analysis (lane 3) of the cross-linked IgG which was electroeluted from the

native gradient gel gave the same three bands as shown in Fig. 1*a*, lane 4. In addition, the relative intensity of the upper band, in relation to the intensities of the heavy and light chain bands, is identical to that obtained in Fig. 1*a*, lane 4. Since under non-denaturing conditions the cross-linked IgG migrated the same distance as the untreated IgG monomer (Fig. 2, lane 1 versus lane 2), the upper band in lane 3 must be the product of intramolecular cross-linking of the two IgG heavy chains through their carbohydrate moieties.

The cross-linking reaction could proceed through the Fc or Fab regions of the heavy chains. To discriminate between these two possibilities, the cross-linked IgG was cleaved by papain. Papain produces two Fab fragments from one IgG molecule, and a cross-link between two heavy chains in the Fab region would result in (Fab)₂ species (see below, Fig. 7*a*). However, a band corresponding to such species is hardly visible, in contrast with the strong Fab band (Fig. 3, lane 2). This result indicates that the cross-linking occurs predominantly, perhaps exclusively, through the Fc part of the heavy chains. It is interesting to note that the cross-linked IgG and its Fc fragments bound to immobilized Protein A (lane 1), which was used to separate Fc and Fab species. The strong band between Fc and the uncleaved IgG (lane 1) is probably an intermediate still containing one Fab piece.

We have also investigated the influence of the degree of oxidation and the cross-linker lengths on the cross-linking. Fig. 4 shows the results of experiments whereby variously oxidized IgGs were incubated with either MADH (*a*) or AADH (*b*). Oxidation conditions comparable with those reported to specifically oxidize sialic acid residues in complex-type sugar chains [23] were not sufficient to give cross-linking by either one of the two dihydrazides (lanes 2). After a longer oxidation, a

Fig. 2. Analysis of cross-linked IgG by non-denaturing electrophoresis in a 3–30% polyacrylamide gradient gel, followed by its electroelution and SDS/PAGE

Native IgG was run in lane 1. IgG that was cross-linked under the conditions specified in Fig. 1 (lane 4) is shown in lane 2. The cross-linked IgG was electroeluted [from the gel piece (hole) as seen in lane 2] and subsequently analysed by SDS/PAGE (lane 3) using the buffer system of Laemmli [15]. 2H denotes the proposed heavy chain dimer.

Fig. 3 Papain cleavage and Protein A chromatography of cross-linked rabbit IgG

A sample of cross-linked rabbit IgG (as specified in Fig. 1, lane 4) was bound to and eluted from a Protein A-Sepharose column. Subsequently it was treated with papain (20:1, w/w) for 3 h at 37 °C. The proteolysis was stopped by adding iodoacetic acid (20 mM final concentration). The sample was then applied to a Protein A column. The unbound as well as the eluted fraction were dialysed and analysed by SDS/PAGE under non-reducing conditions. In lane 1 the cross-linked rabbit IgG, cleaved with papain and bound to and eluted from Protein A column, is shown. In lane 2 the fraction of the same sample that did not bind to protein A is shown. Lane 3 contains an untreated rabbit IgG cleaved by papain (20:1, w/w) for 12 h at 37 °C. All other conditions were as specified in the legend to Fig. 1.

Fig. 4. SDS/PAGE of rabbit IgG oxidized under different conditions and incubated with dihydrazides differing in size

Untreated IgG was applied to lane 0 and oxidized IgG (10 mM- NaIO_4 , 2 h, 0 °C, without dihydrazide) to lane 1. Before cross-linking with 20 mM-MADH [approx. 7 Å (0.7 nm) long] in (a) or with 20 mM-AADH [approx. 12 Å (1.2 nm) long] in (b), IgG was oxidized under sialic-acid-specific conditions, that is at 0 °C with 4 mM- NaIO_4 , for 15 min (lane 2), or for 2 h (lane 3) or 4 h (lane 4). It was also oxidized for 2 h with 40 mM- NaIO_4 (lane 5) or 100 mM- NaIO_4 (lane 6). s_1 and s_2 are molecular mass standards. All other conditions were as in the legend to Fig. 1.

weak upper band appeared (lanes 3). Its intensity increased gradually with longer oxidation or with higher NaIO_4 concentration (lanes 4 and 5). The maximal ratio between the relative intensity of the cross-linked heavy chain dimer and the heavy chain monomer was obtained with 100 mM- NaIO_4 and a 2 h oxidation period (lanes 6). The difference in the link distance of about 5 Å (0.5 nm) between MADH and AADH had no

influence on the quantity of the heavy chain dimer (compare Figs. 4a and 4b). As can be seen, we could never achieve complete cross-linking of the two rabbit IgG heavy chains (Figs 4a and 4b, lanes 6). This is perhaps due to existence of many differently glycosylated IgG forms, some of which may be impossible to cross-link. However, we have observed that heating the cross-linked IgG at 100 °C in the presence of SDS and mercaptoethanol

Fig. 5. SDS/PAGE analysis of cross-linking of IgGs from various species

(a) Human IgG. Oxidized IgG (40 mM- NaIO_4 , 2 h, 0 °C) was applied to lane 1. The oxidized IgG (4 mM- NaIO_4 for 15 min) was cross-linked with AADH (lane 2). It was also more strongly oxidized (40 mM- NaIO_4 , 2 h) and incubated with MADH (lane 3) or AADH (lane 4). Human IgG that was oxidized with 100 mM- NaIO_4 for 2 h and incubated with AADH is shown in lane 5. (b) IgGs of different mammalian species. Two samples of each IgG were run. The gel shows oxidized IgGs (40 mM- NaIO_4 , 2 h, 0 °C) of sheep (lane 1), pig (lane 3), goat (lane 5) and guinea pig (lane 7), and the AADH cross-linked IgGs of sheep (lane 2), pig (lane 4), goat (lane 6) and guinea pig (lane 8). (c) Chicken IgY. The oxidized IgY (10 mM- NaIO_4 , 2 h, 0 °C) is shown in lane 1. The IgY oxidized with 4 mM- NaIO_4 for 15 min at 0 °C and cross-linked with AADH is shown in lane 2. It was also oxidized for 2 h and incubated with MADH (lane 3) or with AADH (lane 4). The IgY oxidized with 40 mM- NaIO_4 for 2 h and incubated with 20 mM-AADH is shown in lane 5. All other conditions were as specified in the legend to Fig. 1.

considerably decreases the intensity of the band corresponding to cross-linked heavy chains (results not shown), indicating that certain cross-links may be cleaved under conditions necessary for complete reduction of disulphide bridges in rabbit IgG.

Human IgG was cross-linked by the same procedure as for rabbit IgG and (Fig. 5a) the cross-linking pattern is comparable with that of rabbit IgG (Fig. 4). Sialic-acid-specific oxidation was again insufficient for cross-linking (lane 2), and there was no difference between the short (MADH) and the long (AADH) cross-linker (lane 3 versus lane 4). Again, it was not possible to achieve complete cross-linking of the two heavy chains (lane 5).

Extensive oxidation was also necessary to achieve cross-linking in sheep (lane 2), pig (lane 4), goat (lane 6) and guinea pig (lane 8) IgGs (Fig. 5b). In all of these species, sialic-acid-specific oxidation was not sufficient to produce cross-linking (results not shown). An exception was chicken 7s immunoglobulin (IgY) (Fig. 5c), which yielded heavy chain dimers after only very weak oxidation (4 mM- NaIO_4 , 15 min, 0 °C) (lane 2). After extensive oxidation, almost all of the heavy chains were cross-linked (lane 5), forming not only dimers but also higher oligomers. These oligomers are most probably the products of intermolecular cross-linking, since an increase of cross-linker concentration to 80 mM greatly decreased oligomer formation (results not shown). As with the other immunoglobulins, MADH in place of AADH (lane 3 versus lane 4) gave the same relative intensity of the heavy chain dimer band.

With the cleavable cross-linker, DTDPADH, it is possible to estimate the number of cross-linker molecules introduced into a glycoprotein by measuring the increase in the number of disulphide bonds (Table 1). The number of the bound cross-linkers clearly depends on the oxidation conditions and, for fetuin, also on the concentration of DTDPADH. Thus IgG which was oxidized under conditions that gave good cross-linking (Fig. 4, lane 5) was found to bind two to three molecules of DTDPADH (Table 1). Invertase, oxidized under conditions reported to be specific for sialic acid residues, bound about 47 DTDPADH molecules. This result indicates that high-mannose-type glyco-

Fig. 6. SDS/PAGE of various glycoproteins cross-linked with AADH or with the cleavable cross-linker, DTDPADH, in the absence (a) or the presence (b) of 2-mercaptoethanol

Lane 1, untreated rabbit IgG; lane 2, rabbit IgG cross-linked with AADH as specified in Fig. 4b (lane 5); lane 3, rabbit IgG cross-linked with DTDPADH (two DTDPADH molecules introduced, see Table 1); lane 4, cross-linked invertase with 67 DTDPADH molecules introduced (Table 1); lane 5, cross-linked chicken IgY with nine DTDPADH molecules introduced (Table 1). Otherwise, conditions were the same as in Fig. 1.

proteins are oxidized extensively under conditions specific for sialic acid in the complex-type glycans.

Three of the samples in Table 1 were analysed further by SDS/PAGE under non-reducing (Fig. 6a) and reducing (Fig. 6b) conditions. Under non-reducing conditions, the protein band pattern of IgG cross-linked with either AADH (lane 2) or DTDPADH (lane 3) is apparently the same, both containing additional weak high molecular mass bands compared with untreated IgG (lane 1, see also Fig. 1b). The cross-linked chicken IgY has a band pattern similar to that of rabbit IgG, except that

Table 1. Amounts of DTDPADH introduced into oxidized immunoglobulins and some other glycoproteins

Incorporation of DTDPADH was calculated from the difference between disulphide groups in the cross-linked and in the native glycoproteins. For calculation of number of DTDPADH molecules in rabbit IgG, two batches of rabbit IgG were cross-linked and analysed several times. To prove that the values were not underestimated due to oxidation of some disulphide bonds by periodate, the number of disulphide bridges was determined also in an oxidized IgG (40 mM- NaIO_4 , 2 h, 0 °C) and in the AADH-cross-linked rabbit IgG. The values obtained were the same as in the native IgG. The cross-linking and analysis of other glycoproteins was done twice with, within the experimental error, identical results.

Protein	Oxidation time (min)	[NaIO_4] (mM)	[DTDPADH] (mM)	Number of DTDPADH molecules introduced
IgG, rabbit	15	4	40	0
	120	40	40	2-3
	120	40	0.2	2-3
IgG, human	15	4	40	0
	120	40	40	3
IgG, guinea pig	15	4	40	0
	135	40	40	2
IgY, chicken	15	4	40	2
	60	40	40	9
Fetuin	15	4	0.2	2
	15	40	40	4
	120	4	0.2	3
	120	4	40	6
Invertase	15	4	40	47
	120	4	40	67

with the finding that the rabbit Fc fragment contains only about 0.26 mol of sialic acid and no disialylated glycans [3]. Moreover, we have incubated the oxidized glycoproteins at two different concentrations of DTDPADH, because the low concentration should favour the reaction of one DTDPADH molecule with two aldehydes, whereas the high concentration of the cross-linker should shift the molar ratio between DTDPADH and aldehydes towards 1 : 1. In rabbit IgG, no difference was observed, which is again an indication for fast and complete intramolecular cross-linking. In fetuin, however, the number of the cross-linker molecules introduced was doubled at the higher cross-linker concentration (Table 1).

How the cross-linking of IgG affects its biological functions remains to be fully investigated. In general, cross-linking reduces the overall flexibility of the molecule, which results in increased stability against heat denaturation and proteolysis in highly glycosylated proteins [10,11]. The introduction of more than 60 cross-linker molecules into invertase (Table 1) rationalizes this finding. This effect may not be so pronounced in the case of IgG, because only two to three cross-linker molecules were introduced. Nevertheless, since upon cross-linking the flexibility of the C_H2 domain is probably partially decreased, those interactions occurring through the C_H2 domain and/or involving the N-linked glycans [9] might be affected. Our results (Fig. 3) show that binding to immobilized protein A is apparently not affected by cross-linking of the glycan chains. However, the Fc region of the periodate-oxidized as well as cross-linked IgG were more susceptible to papain cleavage than the Fc part of native IgG (results not shown).

Cross-linking through the carbohydrate chains is of particular interest for membrane glycoproteins, since their amino acid side chains are less accessible. To take advantage of the fact that their oligosaccharides are readily accessible, membrane glycoproteins have been cross-linked through amino groups introduced into their glycans by periodate oxidation and reductive amination [25]. However, such a procedure requires at least three separate incubation steps. Our procedure requires only two steps, and since the reaction of a hydrazide with an aldehyde is very selective and results in the formation of a stable hydrazone or carbinol hydrazide bond [26], the cross-linking procedure investigated here appears more suitable.

The advantages of cleavable cross-linkers are widely recognized in studies on protein interaction and organization of multi-molecular complexes [27]. The utility of the herein-introduced DTDPADH as a specific and cleavable carbohydrate-carbohydrate cross-linker has been demonstrated clearly (Fig. 6, Table 1). We expect that its major future application will be in the demonstration of the proximity of the carbohydrate chains of glycoproteins situated in subcellular structures and in the identification of those glycan chains that have been cross-linked.

This work was supported in part by the Swiss National Science Foundation, Grant No. 3.436-83. B.K. gratefully acknowledges the fellowship from Procordia Nova.

REFERENCES

1. Rademacher, T. W. & Dwek, R. A. (1984) *Prog. Immunol.* **5**, 95-112
2. Parekh, R. B., Dwek, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, T., Matsuta, K., Takeuchi, F., Nagano, Y., Miyamoto, T. & Kobata, A. (1985) *Nature (London)* **316**, 452-457
3. Taniguchi, T., Mizuochi, T., Beale, M., Dwek, R. A., Rademacher, T. W. & Kobata, A. (1985) *Biochemistry* **24**, 5551-5557
4. Savvidou, G., Klein, M., Horne, C., Hofmann, T. & Dorrington, K. J. (1981) *Mol. Immunol.* **18**, 793-805
5. Homans, S. W., Dwek, R. A., Fernandes, D. L. & Rademacher, T. W. (1984) *Biochim. Biophys. Acta* **798**, 78-83
6. Sutton, B. J. & Phillips, D. C. (1983) *Biochem. Soc. Trans.* **11**, 130-132
7. Deisenhofer, J. (1981) *Biochemistry* **20**, 2361-2370
8. Rademacher, T. W., Homans, S. W., Parekh, R. B. & Dwek, R. A. (1986) *Biochem. Soc. Symp.* **51**, 131-148
9. Burton, D. R. (1985) *Mol. Immunol.* **22**, 161-206
10. Kozulić, B., Barbaric, S., Ries, S. & Mildner, P. (1984) *Biochem. Biophys. Res. Commun.* **122**, 1083-1087
11. Kozulić, B., Leustek, I., Pavlovic, B., Mildner, P. & Barbaric, S. (1987) *Appl. Biochem. Biotech.* **15**, 265-278
12. Curtius, S., Schoefer, A. & Schwan, C. (1895) *J. Prakt. Chem.* **51**, 187
13. Sakamoto, M., Takeda, J., Yamada, Y. & Tonami, H. (1970) *J. Appl. Polym. Sci.* **14**, 865-878
14. Tijssen, P. (1985) in *Practice and Theory of Enzyme Immunoassays* (Burdon, R. H. & van Knippenberg, P. H., eds.), pp. 117-121, Elsevier, Amsterdam
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
16. Bury, A. F. (1981) *J. Chromatogr.* **213**, 491-500
17. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310
18. LeBlanc, G. A. & Cochrane, B. J. (1987) *Anal. Biochem.* **161**, 172-175
19. Thannhauser, T. W., Konishi, Y. & Scheraga, A. H. (1984) *Anal. Biochem.* **138**, 181-188
20. Fasman, G. D. (ed.) (1986) *Handbook of Biochemistry and Molecular Biology*, 3rd edn., CRC Press, Boca Raton
21. Tankersley, D. L., Preston, M. S. & Finlayson, J. S. (1988) *Mol. Immunol.* **25**, 41-48
22. Slater, G. G. (1969) *Anal. Chem.* **41**, 1039-1041
23. Van Lenten, L. & Ashwell, G. (1971) *J. Biol. Chem.* **246**, 1889-1894
24. Benedict, A. & Yamaga, K. (1972) in *Comparative Immunology* (Marchalonis, J. J., ed.) pp. 335-375, Blackwell, Oxford
25. Schweizer, E., Angst, W. & Lutz, H. U. (1982) *Biochemistry* **21**, 6807-6818
26. Hansske, F., Sprinzl, M. & Cramer, F. (1974) *Bioorg. Chem.* **3**, 367-376
27. Peters, K. & Richards, F. M. (1977) *Annu. Rev. Biochem.* **46**, 523-551
28. Bunton, C. A. (1965) in *Oxidations in Organic Chemistry Part A* (Wiberg, K., ed.), p. 367. Academic Press, New York

Received 27 November 1989; accepted 4 January 1990