

# Preparation of Conjugates of Proteins with Amyloses by Elongation of Covalently Attached Primers Using Glycogen Phosphorylase *a*<sup>1</sup>

YEN-HO CHU AND GEORGE M. WHITESIDES<sup>2</sup>

*Department of Chemistry, Harvard University, 12 Oxford Street,  
Cambridge, Massachusetts 02138*

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This paper describes convenient preparations of protein-amylose conjugates. These preparations are based on the elongation of maltooligosaccharides by reaction with glucose-1-phosphate, catalyzed by glycogen phosphorylase *a*. Coupling of maltooligosaccharides to proteins by reductive amination generated covalently attached primers for the glycogen phosphorylase *a*-catalyzed polymerization of glucose-1-phosphate. SDS-polyacrylamide gel electrophoresis was useful for characterizing these conjugates. <sup>31</sup>P NMR spectroscopy could be used conveniently to assay simultaneously the formation and the enzymatic activity of the amylose-conjugated proteins. This assay could be used directly in reactions catalyzed by glycogen phosphorylase *a*; it should also be applicable to protein mixtures of greater complexity than the ones used here. Methods of synthesizing enzymatically active neoglycoproteins having a range of molecular weights are described. © 1993 Academic Press, Inc.

## INTRODUCTION

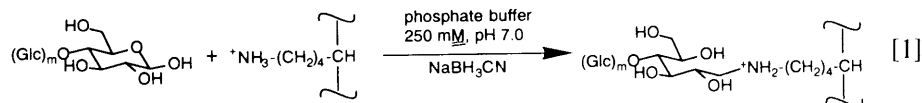
The objective of this work was to develop methods to prepare conjugates of proteins with high molecular weight amyloses. We wished to compare the properties of this class of neoglycoproteins with those of naturally occurring glycoproteins and with conjugates of proteins with poly(ethylene glycol) (PEG) and other man-made polymers. The oligosaccharide moieties of glycoproteins may play a number of roles: maintaining protein conformation and solubility (1); stabilizing the polypeptide against proteolysis (2); functioning in processing, intracellular sorting, and excretion of glycoproteins (3); mediating biological activity (4); and serving as cell-surface labels in differentiation and development (1, 5). Functions depending on biological recognition of sugar groups undoubtedly require specific oligosaccharide structure; functions depending on more general physical properties (solubility, thermal stability, resistance to proteases, and masking against recognition by antibodies) may be replicable using simpler oligosaccharide moie-

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<sup>2</sup> To whom correspondence should be addressed.

ties. Conjugates of proteins with PEG have shown a number of interesting properties and are now being introduced into clinical practice on the basis, *inter alia*, of low immunogenicity and long serum lifetime (6).

Direct attachment of preformed polysaccharides to proteins is difficult for several reasons. It is difficult to functionalize a polysaccharide selectively in a particular position (e.g., a terminal position), difficult to achieve reaction of many polymers with surfaces or high molecular weight reactants once activated, and difficult to characterize the products. We have taken a different approach (Eq. [1]). We



modify the protein of interest by attachment of a low-molecular-weight oligosaccharide, derived from maltohexose (penta[ $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  4)]-D-glucopyranose) or its derivatives. The resulting protein-maltohexose conjugates can be characterized, at least to the extent of establishing the number, even if not the specific location, of attached oligosaccharides. These covalently attached maltohexose derivatives act as primers for the polymerization of glucose-1-phosphate (Glc-1-P) catalyzed by phosphorylase *a* (see below).

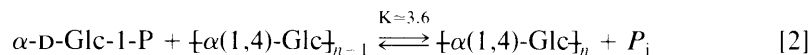
This system has the advantages that the protein-maltohexose conjugates can be characterized and that the *in situ* polymerization catalyzed by phosphorylase *a* is compatible with retention of activity in the protein of interest. The amylose chain attached to the protein can also be shortened with retention of activity by treatment with phosphorylase *a* and phosphate in the absence of Glc-1-P or by hydrolysis with amylase (1,4- $\alpha$ -D-glucan glucanohydrolase, EC 3.2.1.1). Characterizing the protein-amylose conjugates—especially the degree of polymerization of the amylose chains and the number of primers participating in the polymerization reactions—remains a problem that we have only partially solved.

The production of linear and branched amyloses has been developed extensively, especially by Ziegast and Pfannemüller (7). We have described the initial results of our work in an earlier paper (8). We selected cytochrome *c* (from horse heart) (9), ribonuclease A (RNase A, EC 3.1.27.5, from bovine pancreas) (10), and carbonic anhydrase (CA, carbonate hydrolyase, EC 4.2.1.1, from bovine erythrocytes) (11) as model proteins for several reasons. All are commercially available, inexpensive, and well characterized. CA is monomeric, with a molecular weight of 30 kDa. It contains 19 lysine residues, all of which are on the surface of the protein and accessible to the modifying agents. Assaying the catalytic activity of CA is straightforward (11). RNase A is a single polypeptide, with a molecular weight of 14 kDa and has 10 lysine residues. One of these (Lys-41) is at the active site of the enzyme (10). We have developed a simplified assay system for RNase A.<sup>3</sup> Cytochrome *c* is also monomeric, with a molecular weight of 12.4

<sup>3</sup> The assay for activity of RNase using <sup>31</sup>P NMR spectroscopy was developed in our laboratory by Rajeeva Singh.

kDa. It possesses 18 lysines, all on the surface of the protein. Cytochrome *c* has a strong absorption at 409 nm ( $\epsilon_{409}/\epsilon_{280} = 17$ ) and is easily assayed spectrophotometrically (9).

Glycogen phosphorylase (1,4- $\alpha$ -D-glucan:orthophosphate  $\alpha$ -D-glucosyltransferase, EC 2.4.1.1) catalyzes the reversible polymerization of Glc-1-P (Eq. [2]). The best-studied isozyme of phosphorylase is that from



rabbit skeletal muscle (12).<sup>4</sup> The enzyme is highly specific with respect to its substrates. Only  $\alpha$ -D-Glc-1-P reacts in the transfer of a glucosyl residue. This enzyme requires a primer as the second substrate to effect polymerization. Maltotetraose (i.e., tri[ $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  4)-D-glucopyranose]) is the minimum oligomer that will function as primer. The equilibrium constant for the polymerization reaction (Eq. [2]) at neutral pH is 3.6 and favors glycogen synthesis *in vitro* (1, 13).<sup>5</sup>

## EXPERIMENTAL PROCEDURES

### Materials

Proteins and enzymes used were obtained from Sigma (St. Louis, MO): cytochrome *c* (from horse heart), ribonuclease A (from bovine pancreas), carbonic anhydrase (from bovine erythrocytes), sucrose phosphorylase (from *Leuconostoc mesenteroides*), and glycogen phosphorylase *a*. Maltohexose and maltoheptose (i.e., hexa[ $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  4)]-D-glucopyranose) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Maltose (i.e.,  $\alpha$ -D-glucopyranosyl-(1  $\rightarrow$  4)-D-glucopyranose), sodium borocyanohydride, and sucrose were commercial products of Aldrich (Milwaukee, WI). 2',3'-cCMP, 3'-CMP, glucose-1-phosphate, poly(A), and *p*-nitrophenyl acetate were obtained from Sigma. Reagents and apparatus (e.g., mini PROTEAN II) used for polyacrylamide gel electrophoresis (PAGE) were products of Bio-Rad (Richmond, CA). SDS-PAGE was carried out following the manufacturer's procedure.

<sup>4</sup> The most active form of rabbit muscle glycogen phosphorylase is a dimer of two identical monomers, with 842 amino acid residues and a molecular weight of 97,440 Da for each subunit. This enzyme contains a pyridoxal phosphate as a cofactor, covalently bound via a Schiff base to an active site lysine (Lys-680). In the resting state, the enzyme exists as the inactive phosphorylase *b*, which may be activated by AMP. In response to nervous or hormonal stimulation, the *b* form is phosphorylated and becomes the *a* form: phosphorylase *a* is no longer dependent on AMP for activity. The interconversion of phosphorylase *a* and phosphorylase *b* involves the phosphorylation of a single serine residue (Ser-14) by phosphorylase *b* kinase and its dephosphorylation by the enzyme phosphorylase *a* phosphatase (12).

<sup>5</sup> Although the polymerization reaction is favored at neutral pH *in vitro*, the reaction *in vivo* proceeds toward the degradation of glycogen because the intracellular concentration of  $P_i$  greatly exceeds that of Glc-1-P and because Glc-1-P is rapidly converted to Glc-6-P by phosphoglucomutase (13).

#### *Preparation of Cytochrome *c*-Maltoheptose Conjugates*

Conjugation was carried out using a modification of the published procedure (14). In a typical experiment, lyophilized cytochrome *c* (from horse heart) was added to a phosphate buffer (250 mM, pH 7.0). To this protein solution maltoheptose and sodium cyanoborohydride, each dissolved in the same buffer, were added to final concentrations of 10 mg/ml for the protein and 200 and 81 mg/ml, respectively, for maltoheptose and sodium cyanoborohydride. Reaction was carried out at ambient temperature, the reaction mixture was sampled at various reaction times, and the samples were dialyzed and lyophilized. The contents of carbohydrate in these conjugates were determined by the anthrone-sulfuric acid method (15). Concentrations of cytochrome *c* in these conjugates were established spectrophotometrically (409 nm,  $\epsilon = 9.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ) (Table 1).

#### *Preparation of Carbonic Anhydrase-Maltoheptose Conjugates*

The same procedure as that used in the preparation of cytochrome *c*-maltoheptose conjugates was followed, except that concentrations of 3 mg/ml for carbonic anhydrase (from bovine erythrocytes), 50 mg/ml for maltoheptose, and 10 mg/ml for sodium cyanoborohydride were used. The reaction was carried out at room temperature for 18 h and the protein sample was dialyzed against phosphate buffer (2 liters, 5 mM, pH 7.0), water (2 liters) and lyophilized. The ratio of [maltoheptose]/[protein] was determined to be 1.5.

#### *Preparation of Ribonuclease A-Maltooligosaccharide Conjugates*

To a phosphate solution (4 ml, 250 mM, pH 7.0) of RNase A (31 mg, from bovine pancreas) maltohexose (280 mg) and sodium cyanoborohydride (18 mg) were added. The reductive amination was carried out at 4°C for 12 h. The reaction mixture was dialyzed and lyophilized. The average number of maltohexose molecules per RNase A was 1.6, as determined by the anthrone-sulfuric acid method. The same protocol was followed in preparing RNase A-maltose conjugates. The average number of maltose molecules per RNase A was 1.9. The conjugation of lysine residues of RNase A, in the presence of poly(A), was performed with maltohexose according to a modification of the procedure of Blackburn and Gavilanes (16). A protocol the same as that described above was followed except that a ratio [poly(A)]/[RNase A] of 31 was used. After 24 h of reaction, the reaction mixture was dialyzed and lyophilized. The ratio of [maltohexose]/[RNase A] was 4.3.

#### *Assay of Ribonuclease A for Activity*

The normal uv assay used for the activity of RNase A was not straightforward and could not be used for mixtures of glycogen phosphorylase *a* and conjugated RNase A since the  $\epsilon_{284 \text{ nm}}$  ( $1.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ), between 2',3'-cCMP and 3'-CMP, varied with concentrations of phosphorylase *a* and polysaccharides. The  $^{31}\text{P}$  NMR assay of RNase A (developed by Rajeeva Singh) is simple and useful in that it directly quantitates both the substrate 2',3'-cCMP and the product 3'-

CMP. The activity of RNase A was determined by following the conversion of 2',3'-cCMP to 3'-CMP (Fig. 2).

For a typical assay, a solution of 2',3'-cCMP (10 mM) in MOPS buffer (0.1 M, pH 7.0) was placed in a NMR tube, its  $^{31}\text{P}$  NMR spectrum was recorded, and then a solution of RNase A (typically 6  $\mu\text{M}$ ) was added to a final volume in the tube of 1.5 ml. The time course of the conversion of 2',3'-cCMP to 3'-CMP was followed and the activity of RNase A determined. Without RNase A, there was <2% hydrolysis of 2',3'-cCMP in buffer over the course of the assay (about 4.5 h).

#### *Assay of Carbonic Anhydrase*

The procedure of Armstrong *et al.* (17) was used to assay the activity of CA in this study: *p*-nitrophenyl acetate was used as the substrate and the activity of CA was followed spectrophotometrically by the production of *p*-nitrophenolate ( $\epsilon_{348\text{ nm}} = 5.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).

#### *Assay for the Formation of Protein-Amylose Conjugates*

We have developed a simple  $^{31}\text{P}$  NMR system for assaying glycogen phosphorylase *a*-catalyzed reactions. At neutral pH, signals of Glc-1-P and  $P_i$  in a  $^{31}\text{P}$  NMR spectrum are well separated (Fig. 2). Typical conditions for an assay were Glc-1-P, 83 mM; protein-maltooligosaccharide conjugates, 5.5 mM; and glycogen phosphorylase, 18 units, in MOPS buffer (0.2 M) at pH 7.0 with a total volume of 3.3 ml. The assay was carried out by following the disappearance of Glc-1-P and the appearance of  $P_i$  formed.

The formation of protein-amylose conjugates would also be followed by gel electrophoresis under denaturing condition (SDS-PAGE).

#### *Estimation of Molecular Weights of Protein-Amylose Conjugates*

The gel-filtration HPLC was used for estimating molecular weights of protein-amylose conjugates enzymatically synthesized by glycogen phosphorylase *a*: column, Waters Protein Pak 300 (0.75  $\times$  30 cm); mobile phase, double-distilled water; detection, 254 nm; flow rate, 0.5 ml/min.

## RESULTS AND DISCUSSION

#### *Conjugation of Maltooligosaccharides with Proteins*

We surveyed a number of methods for conjugation of maltooligosaccharides with proteins (18). We concluded that reductive amination of proteins with maltooligosaccharide using sodium cyanoborohydride is best; coupling of bromine-oxidized maltooligosaccharides with proteins—using activation of the C-1  $\text{CO}_2\text{H}$  group with water-soluble 1-ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC)—is also useful. Reductive amination preserves protein structure well (Eq. [1]) (18). The reaction converts lysine  $\epsilon$ -amino residues and the

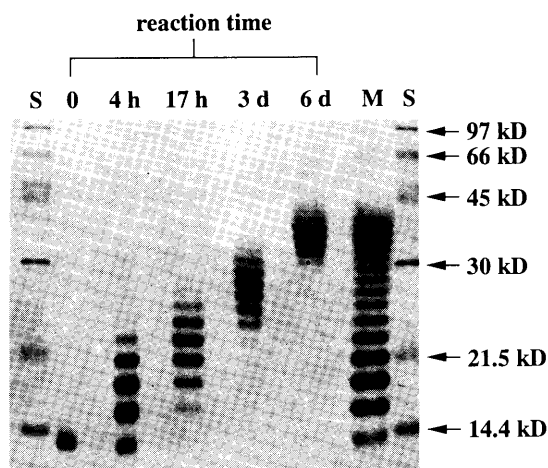


FIG. 1. Sodium dodecyl sulfate-denatured polyacrylamide gel (18% T, 0.5% C) electrophoresis of cytochrome *c*-maltoheptose conjugates synthesized by reductive amination in the presence of sodium borocyanohydride and isolated by dialysis and lyophilization. Conjugation of maltoheptose to cytochrome *c* ([maltoheptose]:[cytochrome *c*] = 215:1) was carried out at room temperature (pH 7.0, 250 mM phosphate buffer) for various reaction times. Lane M is a mixture of cytochrome *c* conjugates prepared using various reaction times, and lane S is a set of protein MW standards; h, hours; d, days. The gel was stained using Coomassie brilliant blue R-250.

N-terminal amino group to secondary amines: the positive charge present on these amino groups is retained.

#### *Characterization of Maltooligosaccharide Conjugates of Proteins*

We determined the carbohydrate content of the modified proteins using the anthrone-sulfuric acid method with glucose as a standard (15). SDS-PAGE was useful for analyzing the extent of conjugation of the proteins (19). After conjugation of cytochrome *c* with maltoheptose, we observed 18 distinct species in the SDS-PAGE gel (Fig. 1).<sup>6</sup> We believe that these species correspond to native cytochrome *c* and conjugates containing from 1 to 17 lysines modified by reductive amination. The N-terminal glycine residue of cytochrome *c* is post-translationally blocked by acetylation (9). Most of lysine residues on horse heart cytochrome *c* could be reductively aminated with maltoheptose under conditions mild enough to be nondenaturing (250 mM phosphate buffer, pH 7.0).

Table I summarizes three groups of protein conjugates that we have synthesized. Conjugates of RNase A and CA with oligomaltoses retained activity satisfactorily

<sup>6</sup> SDS-PAGE is broadly used for determining molecular weights of proteins (22). We plotted the relative electrophoretic mobilities as a function of the calculated molecular weights of these cytochrome *c*-maltoheptose conjugates and yielded no reliable information on their molecular weights. It is known from the literature (23) that the polyacrylamide gel electrophoretic behavior of glycoprotein-SDS complexes yields abnormal estimates of their molecular weights.

TABLE I

Conjugation of Maltooligosaccharides (Glc<sub>n</sub>) to Cytochrome *c* (Cyt *c*), Ribonuclease A (RNase A), and Carbonic Anhydrase (CA) by Reductive Amination, Using Sodium Cyanoborohydride as the Reducing Agent, at pH 7.0

Protein (NH <sub>2</sub> groups)	Glc <sub>n</sub> ( <i>n</i> )	Reaction time (h)	$\frac{[\text{Glc}_n]^b}{[\text{protein}]}$	% Activity remaining
Cyt <i>c</i> (18)	7	4	2.4	
		17	4.0	
		48	7.2	
		72	7.5	
		96	8.2	
		120	8.7	
		144	11	
		264	14	
RNase A(10)	6	12	1.6	26
		24	4.3	85
		12	1.9	30
CA(19)	7	18	1.5	95

<sup>a</sup> The conjugation was carried out in a reaction mixture that contained poly(A) (average molecular weight >100 kDa). See Experimental Procedures for optimized conditions.

<sup>b</sup> Concentrations of maltooligosaccharides were estimated by the colorimetric anthrone-sulfuric acid method ( $\epsilon_{620\text{ nm}} = 0.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ) (15). Absorbances at 409 or 280 nm were used for measuring concentrations of cytochrome *c* ( $\epsilon_{409\text{ nm}} = 9.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ), RNase A ( $\epsilon_{280\text{ nm}} = 0.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ), and CA ( $\epsilon_{280\text{ nm}} = 5.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ). Uncertainties in these average values were estimated to be  $\pm 1.2$ .

under appropriate conditions (85 and 95%, Table 1). We found that active site lysines of RNase A were protected from modification by poly(A), in agreement with the report of Blackburn and Gavilanes (16). In the case of cytochrome *c*-maltoheptose conjugates, the average value of the ratio [maltoheptose]/[cytochrome *c*] (that is, the average number of maltoheptose chains conjugated to each cytochrome *c*) estimated by the anthrone-sulfuric acid method is, qualitatively, in agreement with that estimated from SDS-PAGE (Fig. 1). All protein conjugates we have prepared were able to initiate polymerization catalyzed by glycogen phosphorylase *a* (see below), although probably only some of the chains in highly modified proteins participated.

*Using <sup>31</sup>P NMR Spectroscopy to Assay Simultaneously the Formation and Activity of Amylose-Conjugated Proteins (Neoglycoproteins), in Reactions Catalyzed by Glycogen Phosphorylase a*

Glycogen phosphorylase *a* transfers Glc- $\alpha$ (1,4) units to the nonreducing end of the primer. The primer, in turn, must have at least four glucose units (12). Our initial concern was to prepare derivatives of proteins possessing a covalently attached primer moiety that was recognized by glycogen phosphorylase *a*. We

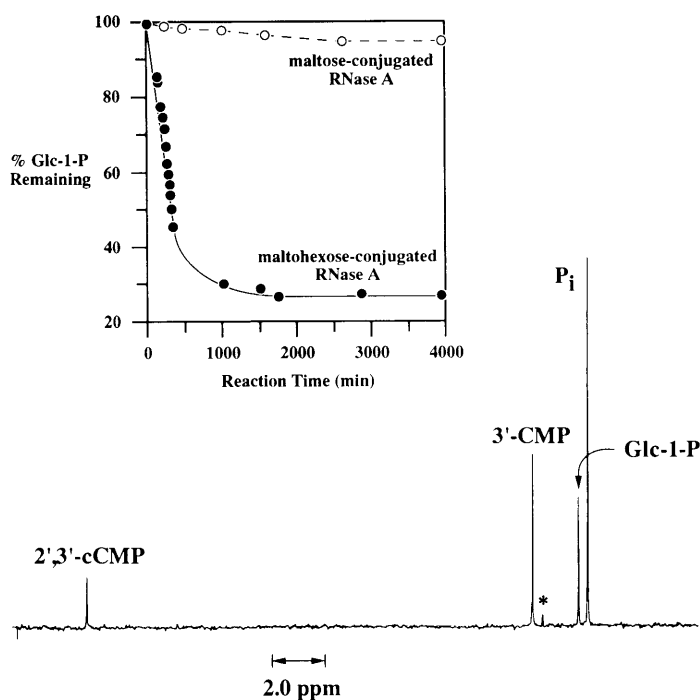


FIG. 2. The phosphorus NMR spectrum of a mixture of  $\alpha$ -D-glucose-1-phosphate (Glc-1-P), inorganic phosphate ( $\text{P}_i$ ), cytidine-3'-monophosphate (3'-CMP), and cytidine-2',3'-cyclic monophosphate (2',3'-cCMP) in 0.2 M MOPS buffer at pH 7.0. The peak marked with an asterisk (\*) was  $\beta$ -glycerophosphate, which was included as a component of the buffer used in commercial preparation of glycogen phosphorylase *a* from rabbit muscle. The consumption of 2',3'-cCMP (substrate) and the production of 3'-CMP (product) establish the biological activity of ribonuclease A (RNase A). Using the same reaction mixture in the same NMR tube, the progress of enzymatic polymerization of saccharide-conjugated RNase A was monitored quantitatively by assaying the conversion of Glc-1-P (substrate) to  $\text{P}_i$  (product), catalyzed by glycogen phosphorylase *a* (Eq. [2]). The inset shows that  $^{31}\text{P}$  NMR spectroscopy can be used to follow the reactions of Glc-1-P (83 mM) with maltohexose (●)- and maltose (○)-conjugated RNase A (5.5 nM; [maltosaccharide]/[RNase A] = 1.6 and 1.9, respectively), catalyzed by glycogen phosphorylase *a* (18 units) in 0.2 M MOPS buffer (pH 7.0), by measuring the remaining Glc-1-P and by the appearance of  $\text{P}_i$ . The maltohexose conjugate reacts rapidly; the maltose conjugate is essentially unreactive.

treated these protein-maltooligosaccharide conjugates with Glc-1-P and phosphorylase *a* and followed the reaction by an  $^{31}\text{P}$  NMR-based assay (Fig. 2).  $^{31}\text{P}$  NMR spectroscopy is useful not only for assaying the process of enzymatic polysaccharidization catalyzed by glycogen phosphorylase *a* but also for determining the enzymatic activity of RNase A. Under appropriate conditions (e.g., 0.2 M MOPS, pH 7.0) we assayed the progress of enzymatic polymerization of protein-conjugated primer quantitatively by monitoring the consumption of Glc-1-P and the production of  $\text{P}_i$ . Using the same reaction mixture in the same NMR tube, the rate of conversion of the substrate 2',3'-cCMP to the product 3'-CMP established the activity



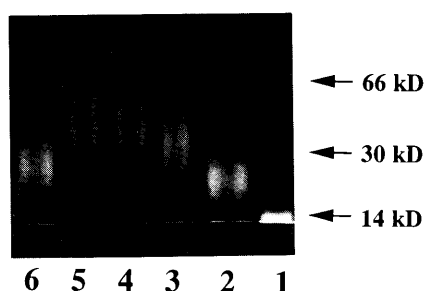


FIG. 3. SDS-PAGE (12% T, 0.3% C) of fluorescent conjugates of ribonuclease A (RNase A) with maltohexose and amylose. Lane 1 is a dansyl-labeled RNase A; lane 2 is the dansylated maltohexose-RNase A conjugate ([maltohexose]/[RNase A] = 4.3); lanes 3-5 are dansylated amylose-RNase A conjugates synthesized by the phosphorylase *a* (5 units)-catalyzed reaction of Glc-1-P (20 mM) and the maltohexose-RNase A conjugate at various reaction times (1 h, for lane 3; 1.5 h, for lane 4; 2 h, for lane 5). Lane 6 is the fluorescent RNase A conjugate obtained by treating the reaction mixture analyzed in lane 5 with  $P_i$  (20 mM) for another 2 h (Eq. [2]).

of the resulting neoglycoRNase A.<sup>7</sup> Figure 2 (inset) also demonstrates that glycogen phosphorylase *a* readily recognized maltohexose-conjugated RNase A as the primer and catalyzed the formation of neoglycoRNase A. As expected, the maltose-RNase A conjugates (used as an experimental control) were not substrates.

These results obtained by <sup>31</sup>P NMR spectroscopy were confirmed by SDS-PAGE using a maltohexose conjugate of RNase A that had been independently labeled with fluorescent dansyl chloride. Fluorescent RNase A-amylose conjugates having higher molecular weights, with respect to their primer, were formed by a phosphorylase *a*-catalyzed reaction of a maltohexose-RNase A conjugate with Glc-1-P (Fig. 3). The recognition of the primer by the phosphorylase *a* was efficient: no free primer was observed after enzymatic polymerization. The molecular weight (~45 kDa) estimated from the SDS-PAGE gel<sup>6</sup> in Fig. 3 corresponds to the addition of 45 glucose units on average to each primer. Adding excessive  $P_i$  to the reaction mixture containing phosphorylase *a* and the amylose-RNase A conjugate (i.e., the reverse reaction of Eq. [1]) decreased the molecular weight of the conjugate to approximately its original value (Fig. 3, lane 6).

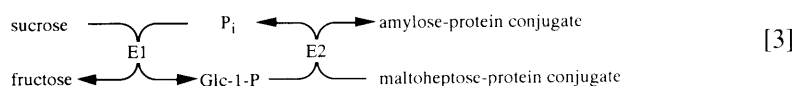
#### *Methods of Producing Enzymatically Active Neoglycoproteins Having Higher Molecular Weights*

Two methods were used for producing neoglycoproteins with higher molecular weights than those obtained by the simple procedures already described. The first used membrane-enclosed enzymatic catalysis (MECC) (20): In the first cycle of

<sup>7</sup> Since RNase A does not lose its enzymatic activity on heating at 100°C for 10 min (10) but glycogen phosphorylase *a* is denatured at this temperature (12), in principle the activity of these RNase A-amylose conjugates at various degrees of polymerization can be determined by heating the phosphorylase *a*-catalyzed reactions.

polymerization, a dialysis tube containing glycogen phosphorylase *a* and the primer protein–oligosaccharide conjugate was placed into a buffer solution of Glc-1-P.  $^{31}\text{P}$  NMR spectroscopy was used to determine whether the reaction achieved equilibrium (Eq. [2]); we then removed the dialysis tube and placed it into another freshly prepared solution of Glc-1-P to achieve further polymerization to synthesize higher molecular weight polymers. By repeating this procedure, it was possible to obtain amylose–protein conjugates with modest molecular weight<sup>8</sup>: we were able to synthesize a neoglycoRNase A with an average molecular weight of 68 kDa, determined by gel-filtration HPLC. This molecular weight indicates that the polymerization had added approximately 150 glucose units to the primer ([maltohexose]/[RNase A]  $\sim 2$ , Table 1).

The second method used an economical enzyme-coupled system for producing higher molecular weight neoglycoproteins (Eq. [3]).



In this equation, E1 and E2 are sucrose phosphorylase (EC 2.4.1.7) (21)<sup>9</sup> from *L. mesenteroides* and glycogen phosphorylase *a* from rabbit muscle, respectively. By using sucrose (in large excess) and inorganic phosphate (in catalytic amount) as starting materials, we synthesized RNase A–amylose conjugates with an average molecular weight of 110 kDa, determined by gel-filtration HPLC. This value corresponds to the addition of 260 glucose units on average to each primer ([maltohexose]/[RNase A]  $\sim 2$ , Table 1).

In conclusion, glycogen phosphorylase *a* from rabbit muscle has been shown to catalyze the synthesis of protein–amylose conjugates from their corresponding primers. This enzyme accepted as substrates the protein–maltooligosaccharide conjugates prepared in this study. Methods of synthesizing and assaying enzymatically active protein–amylose conjugates having higher molecular weights have been developed. These methods provide synthetic routes to a new class of neoglycoproteins and open these materials for examination.

<sup>8</sup> We initially observed no enzymatic catalysis using this method and found that glycogen phosphorylase *a* (from rabbit muscle) adsorbed tenaciously onto the cellulose acetate-based dialysis membrane (e.g., results of X-ray photoelectron spectroscopy showed a high nitrogen content on this nitrogen-free dialysis membrane after exposure to the enzyme). We later demonstrated that the phosphorylase *a* readily catalyzed the polymerization reaction under sonication. Although we have no definitive evidence that defines the role of the sonication, it appeared to desorb the enzyme from the membrane without denaturing the enzyme. We also found that ultrasound generally accelerated enzyme-catalyzed reactions in the MEEC procedure; similar results were observed with acylase I,  $\alpha$ -glucosidase, hexokinase-creatine kinase,  $\beta$ -galactosidase, invertase, sucrose phosphorylase, and glucogen phosphorylase. We presume these rate accelerations are due to the increase of diffusion of substrates and products across the dialysis membrane.

<sup>9</sup> The equilibrium constant for the sucrose phosphorylase-catalyzed reaction at pH 7.0 is 15.6, favoring the synthesis of Glc-1-P (21).

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