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Note

Direct ^{19}F NMR titration of phosphorylase molecules binding to fluorine-labelled glycogen particles

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Glycogen is the principal storage form of carbohydrate in mammals and consists of chains of 11–14 α-(1 → 4)-linked D-glucose residues interconnected by α-(1 → 6)-linked branch points[1]. Glycogen phosphorylase, the major enzyme found bound to glycogen in vivo, cleaves the α-(1 → 4) glucosidic linkages at the nonreducing termini producing α-D-glucopyranosyl phosphate, which is then metabolised. Each glycogen phosphorylase monomer has two glycogen-binding sites, the catalytic site and a higher-affinity glycogen "storage" site. This latter site is involved in enzyme regulation and in binding of the enzyme to the glycogen particles. Crystallographic studies using maltoheptaose bound at the glycogen-storage site have been used to model the phosphorylase–glycogen complex[2]. However, little has been learned of the local dynamics of glycogen in its complex with the enzyme or of the number of enzyme molecules associated with a single glycogen particle.

The synthesis of a glycogen derivative in which all the nonreducing terminal sugars are 4-deoxy-4-fluoro-glucosyl residues (here termed "4-F-glycogen")[3] has allowed a further look at the phosphorylase–glycogen interaction in solution. This glycogen analogue has been shown to bind to phosphorylase, but not to act as a substrate in the direction of glycogen synthesis (i.e., in the presence of α-D-glucopyranosyl phosphate)[3]. This is expected, since the hydroxyl group of the glycogen which acts as the nucleophile in the reaction has been replaced by fluorine. Interestingly however, 4-F-glycogen binds to phosphorylase some 100-fold more tightly than does normal glycogen[3], although the basis of this improved binding is not yet understood. The presence of an NMR-active nucleus (^{19}F) in the molecule should make it possible to monitor the binding interaction between phosphorylase and glycogen, allowing measurements of the stoichiometry of interaction and possibly providing insights into the mode of binding. This technique, which may prove useful for other carbohydrate-binding proteins, is investigated in this work,

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and has allowed estimates to be made of the number of phosphorylase dimers that can bind simultaneously to a single glycogen particle.

RESULTS AND DISCUSSION

Titration of terminal 4-F-glycogen with glycogen phosphorylase was achieved by sequential addition of aliquots of enzyme to a sample of the modified glycogen in a 5-mm NMR tube and acquisition of ^{19}F NMR spectra after each addition. This experiment was performed at two different constant concentrations of 4-F-glycogen (0.6 and 1.2%) and representative spectra from one such titration are shown in Fig. 1.

As may be seen, no significant change in the chemical shift (δ −121.6 ppm) was observed as the enzyme concentration was increased, nor did any new resonances appear. Further, the total peak-area, as determined by integration, remained approximately constant throughout the series. There was, however, a progressive decrease in peak height with increasing enzyme concentration up to a limiting point beyond which no further decrease was observed. Some broadening of the line was also observed as the enzyme concentration increased, linewidths changing from $\Delta\nu_{1/2} = 88$ Hz in the absence of enzyme to $\Delta\nu_{1/2} = 135$ Hz in the presence of saturating concentrations. These observations are consistent with progressive binding of the enzyme to 4-F-glycogen particles until the surface of the particles is completely covered, when no further binding can occur.

It is not clear, however, why the peak height changes, but not the total area, since the increase in linewidth is smaller than the decrease in peak height. The most likely explanation is that, as the observed signal decreases in intensity, a new, broadened component grows into the spectrum at a similar chemical shift. This broad component is not readily apparent, but is nonetheless detected in the integration. Indeed some evidence for such a component can be obtained by comparison of spectra in Figs. 1 b, c, d, and e with that in Fig. 1a, since the peak seems to have a significantly broader base when enzyme is present. Such a broadened component might well be expected if binding of enzyme decreased the local mobility of the fluoro sugar at the end of its chain significantly such that it now acquired the effective correlation time of the total glycogen–enzyme complex rather than the much shorter correlation time of a locally mobile sugar residue attached to the glycogen particle.

It therefore appears that the 4-F glycogen ^{19}F signal obtained in the presence of enzyme results from a combination of "free" and "bound" species. The "bound" population represents 4-fluoro-glucosyl residues which are either directly bound to the protein or are very close in space to bound protein. These probably give rise to a broad component in the ^{19}F signal which unfortunately is not readily apparent. The "free" 4-fluoro-glucosyl residues, present within the [$(\text{phosphorylase})_n$–glycogen] macromolecule, are those whose mobility is affected very little by the binding of the protein, but which are sterically occluded from interaction with other

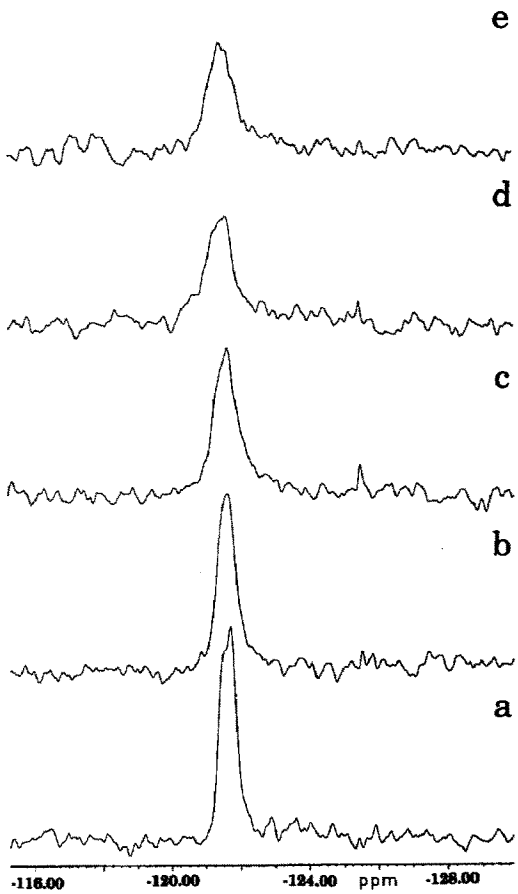

Fig. 1. Titration of 4-F-glycogen with rabbit-muscle glycogen phosphorylase followed by ^{19}F NMR. Conditions: 1.2% 4-F-glycogen, 2.1 mM AMP (initially), 2.3 mM 2-fluoro-D-glucal (initially), 75% D_2O TEA buffer, pH 6.8 (as described in the Experimental section). A constant 4-F-glycogen concentration was maintained while the phosphorylase concentration was increased. Only 5 of the original 10 spectra are shown and these contained the following concentrations of glycogen phosphorylase; (*a*) 0, (*b*) 0.054; (*c*) 0.10; (*d*) 0.15; and (*e*) 0.22 mM.

phosphorylase molecules, and these make up the major component of the ^{19}F signal. Since the increase in linewidth upon enzyme binding indicated that the spin–spin relaxation time (T_2) for this resonance was decreased by the binding of phosphorylase (although this could also be due to chemical-shift heterogeneity), the spin–lattice relaxation time of the resonance observed in the presence of saturating enzyme was also determined in order to assess possible changes in mobility of the fluoro sugars giving rise to this resonance. A value of $T_1 = 0.24$ s was measured for this signal from a sample of 4-F-glycogen (1.2%) containing sufficient phosphorylase (0.22 mM) to completely coat the glycogen particle. This value is slightly smaller than that for free 4-F-glycogen ($T_1 = 0.36$ s), but the fact that only a small decrease has occurred indicates that there is still considerable

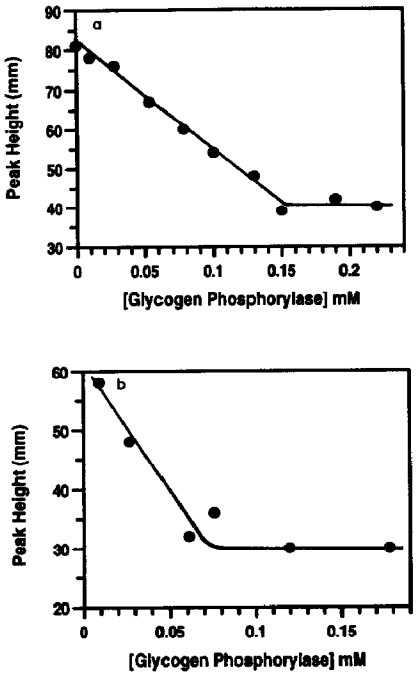

Fig. 2. Plot of peak height of ^{19}F NMR signals versus concentration of phosphorylase added. (Conditions are as described in the Experimental section.) (a): 1.2% 4-F-glycogen, 2.0 mM AMP, and 2.0 mM 2-fluoro-D-glucal; (b) 0.6% 4-F-glycogen, 2.0 mM AMP and 2.0 mM 2-fluoro-D-glucal.

local motional freedom. Indeed, such a difference might well be expected even in the absence of any changes in local motion, given that the correlation time for the [(phosphorylase)$_n$–4-F-glycogen] complex would be considerably larger (that is, a lower rate of reorientation) than that for 4-F-glycogen itself.

As noted previously, peak heights decreased progressively with increasing concentration of phosphorylase up to a point beyond which no further decrease was observed. Fig. 2a shows a plot of peak height versus concentration of phosphorylase (expressed as monomers of molecular weight 97400) using a 4-F-glycogen concentration of 1.2%. Peak height clearly decreases monotonically down to a phosphorylase concentration of 0.15 mM, with no further decrease beyond that point. Such behaviour is suggestive of very tight binding of 4-F-glycogen to phosphorylase (K_D for 4-F-glycogen binding to phosphorylase $\ll 1.2\%$), a result which is consistent with the tight binding observed in previous kinetic studies[3]. A similar set of NMR spectra was accumulated using a 4-F-glycogen concentration of 0.6%, and the peak heights for these ^{19}F signals are shown in Fig. 2b. In this case, peak height decreased to a limiting phosphorylase concentration of ~ 0.07 mM, with no further change beyond that point. Gratifyingly, both sets of data revealed an equivalence point of ~ 0.12 mM phosphorylase for titrating a 1% 4-F glycogen solution. From this it is trivial to calculate the number of phosphorylase molecules

bound per 4-F-glycogen particle. Assuming a molecular weight of 1×10^7 Da for rabbit-liver glycogen[1], then a 1% solution of glycogen corresponds to 1 μM glycogen particles. Since a 1% solution of 4-F-glycogen is titrated by 120 μM glycogen phosphorylase (expressed as monomers), then an average of 60 glycogen phosphorylase dimers (the oligomeric form of the enzyme known to associate with the enzyme) must be bound per glycogen particle. A similar value for the number of phosphorylase molecules bound on a glycogen particle has been determined previously[6] using corn phytoglycogen of very high molecular weight (2×10^7 Da), which can be sedimented in an ultracentrifuge (mammalian glycogen is too small to sediment efficiently). Determination of the amount of phosphorylase co-sedimented at different glycogen:phosphorylase ratios allowed the number of equivalents bound to be determined. A value of 33 phosphorylase molecules of molecular weight 500000 (as thought at that time) per corn phytoglycogen particle was determined. Recalculation of these earlier data in light of the known monomer molecular weight of 97400 would give a total of 85 dimers per phytoglycogen particle. This value is close to that of 60 dimers per particle determined in this work, when the difference in molecular weight (and thus of surface areas) of phytoglycogen and mammalian glycogen particles, is considered.

Interestingly, a glycogen particle of molecular weight 1×10^7 Da will have some 5000 glucose end-groups[6] each of which will, in this case, bear a fluorine label. However, only 60 dimers will be bound to this glycogen particle, providing at best only 240 binding sites (one storage site and one active site per enzyme monomer). Thus a maximum of only 4.8% of the available glycogen end groups will be directly bound to phosphorylase molecules, yet clearly the ^{19}F peak height decreases by a much greater percentage than this. This indicates that the mobilities of many more glycogen chain ends are decreased significantly upon binding of phosphorylase, even though they are not directly bound to the protein. This is quite reasonable, given the highly branched structure of glycogen and the physical interference with motion likely afforded by binding of this relatively large enzyme.

This study has, therefore, demonstrated the utility of ^{19}F NMR in monitoring the binding of proteins to fluorinated polysaccharides. In particular it has provided a direct method for titrating glycogen-binding proteins and determining the stoichiometry of such interactions.

EXPERIMENTAL

Materials.—The 4-F-glycogen derivative (glycogen having all terminal nonreducing D-glucosyl groups replaced by 4-deoxy-4-fluoro-D-glucosyl) was synthesized as previously described[3]. Rabbit-muscle glycogen phosphorylase b was prepared by the method of Fischer and Krebs[4] using (+/-)-1,4-dithiothreitol (DTT) instead of L-cysteine and recrystallized at least three times before use. All other buffer chemicals were purchased from Sigma.

Spectra.—^{19}F NMR spectra were recorded at room temperature on a Bruker AC-200E [quad. nuclei probe (Q.N.P.)] spectrometer operating at 188 MHz with a 5-mm probe. All chemical samples were dissolved in TEA buffer, pH 6.8 (100 mM KCl, 50 mM triethanolamine, 1 mM EDTA, 1 mM DTT) containing 75% D_2O. Glycogen phosphorylase was dialysed for 48 h at 4°C against two changes of TEA buffer, pH 6.8) to remove AMP, and then was further dialysed against the same buffer containing 75% D_2O. The protein was concentrated using a Millipore CL filter unit (30000 Da cutoff). The D_2O used in the NMR studies was previously treated with Chelex (5 mg mL^{-1}) to remove any paramagnetic impurities. Chemical shifts are quoted relative to CF_3CO_2H (δ 0 ppm), and 2-fluoro-D-glucal (δ −92.3 ppm) was used as the internal standard in all experiments. This internal standard was chosen because its chemical shift is close to that of 4-F-glucose, it has no strong fluorine–proton couplings, and it has been shown[7] to be inert to phosphorylase and not to bind at the concentration employed. All experiments were conducted with proton coupling, employing a $35–40^0$ pulse angle, a repetition time of 1.2 s, and a sweep width of 20000 Hz. Exponential line broadening (25 Hz) was used prior to Fourier transformation. Measurements of (T_1) values were performed using the progressive saturation method[5].

Titrations.—Titrations were performed by the sequential addition of glycogen phosphorylase (0.97 mM) to a sample (0.52 mL) of 4-F-glycogen (1.2 or 0.6%) containing, 2.1 mM AMP and 2.3 mM 2-fluoro-D-glucal in 75% D_2O buffer. The 4-F-glycogen concentration was kept constant as the volume increased by the addition of small aliquots (3–8 μL) of concentrated (5.75%) 4-F-glycogen. Such constancy in concentration made subsequent data-analysis considerably more simple. ^{19}F NMR spectra (256 scans) were recorded after each addition of enzyme and compared after setting the absolute intensity of the first free induction decay to 1.

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