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Inactivation and Thermal Stabilization of Glycogenin by Linked Glycogen

Jorge M. Romero, María E. Carrizo, Guillermo Montich, and Juan A. Curtino[1]
Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), UNC-CONICET, Departamento de Química Biológica Dr. Ranwel Caputto, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina

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Glycogen-free but not glycogen-bound glycogenin transglucosylates dodecyl-β-maltoside. Furthermore, its sugar nucleotide-binding site can be photoaffinity labeled using [β-^{32}P]5-azido-UDP-glucose. Disruption with DMSO of the hydrogen bonds that stabilize the α-helical structure of glycogen restored the photoaffinity labeling of the glycogen-bound enzyme but not its transglucosylation activity. The larger size polysaccharide that linked to glycogenin allowed transglucosylation corresponding to that of PG-200, a proteoglycogen species of M_r 200 kDa. PG-200 showed lower activity and increased activation energy than glycogen-free glycogenin. Heat denaturation of glycogen-free and glycogen-bound glycogenin occurred at 51 and 64°C, respectively. Active glycogenin was recovered after the glycogen-bound form was heated at 60–70°C and immediately cooled. Treatment at 60°C of the glycogen-free enzyme resulted in inactivation. This is the first report describing the inactivation and thermal stabilization of an enzyme by linked polysaccharide.

Key Words: **glycogen-bound enzyme; proteoglycogen; glycogenin; transglucosylation activity; thermal stability; photolabeling.**

The effect of glycosylation on the thermal stability and conformation of proteins has been described for several glycoproteins, whose carbohydrate content varied from 2 to 50% (1, 2). Deglycosylation produces a small decrease in denaturation temperature, ranging from 1.3 to 1.8°C in those having a carbohydrate content of about 20% to 2.8°C in yeast external invertase, a glycoprotein having 50% carbohydrate. Deglycosylation did not modify either the secondary structure or the enzyme activity of a dozen glycoproteins, including the highly glycosylated invertase (2).

Proteoglycogen might be considered a particular type of glycoprotein whose protein moiety, glycogenin, amounts to about 1% of its weight (3, 4). Glycogenin is a glucosyltransferase of M_r 38 kDa that can be easily isolated under glycogen-free form, or linked to glycogen or to a polysaccharide of lower size than native glycogen, constituting proteoglycogen and PG-200 (5), respectively. Thus, proteoglycogen offers the possibility of studying the influence of the linkage of a same polysaccharide but of different sizes on a protein. It is expected that maximal changes in the stability and structure of a glycoprotein should be observed at a high carbohydrate content. Any subtle structural modification in the protein moiety of proteoglycogen should be detected by monitoring conformational dependent properties as binding of the substrate and catalytic activity.

Proteoglycogen is the final product of the *de novo* biosynthesis of glycogen, which initiates with the autoglucosylation of its protein moiety, glycogenin (6–8). Glycogenin can autoglucosylate and transglucosylate exogenous acceptors (5, 9–11). Analysing the course of the simultaneous autoglucosylation of glycogenin and transglucosylation of dodecyl-β-maltoside (DBM), we found that the transglucosylation reaction continued even when the acceptor capacity for autoglucosylation was exhausted (5). This result led us to consider the possibility that under the glycogen-bound form, the enzyme could transglucosylate DBM. However, proteoglycogen was inactive. It can be proposed that the large carbohydrate moiety could induce a conformational modification of the enzyme distorting its active site. On the other hand, it could be argued that the large network of oligoglucan chains of glycogen could inhibit the reaction by hindering the access of substrates to the active site of glycogenin.

In order to study the influence of the glycogen moiety of proteoglycogen on the accessibility of substrates to

[1] To whom correspondence and reprint requests should be addressed at CIQUIBIC-Dto. de Química Biológica "Dr. Ranwell Caputto," Facultad de Ciencias Químicas (U.N.C.), Ciudad Universitaria, 5000 Córdoba, Argentina. Fax: 54-351-4334074. E-mail: jcurtino@dqbfcq.uncor.edu.

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glycogenin, we have studied the quenching of the protein intrinsic fluorescence of proteoglycogen by acrylamide, its susceptibility to trypsin digestion and its photoaffinity labeling with [β-^{32}P]5-azidouridine 5′-diphosphoglucose. The effect of linked polysaccharide on the catalytic activity of glycogenin was analysed by comparing the activation energy for transglucosylation of glycogen-free glycogenin with that of the active proteoglycogen species PG-200. We also studied the influence of linked glycogen on the thermal stability of the enzyme by analysing its UV absorption spectrum and transglucosylating activity.

Our results indicate that glycogen attachment increases the thermal stability of glycogenin. The glycogen-bound enzyme can not be photolabeled with [β-^{32}P]5-azidouridine 5′-diphosphoglucose, although the studies carried out with acrylamide and trypsin indicate that the access of substrates to glycogenin is not hindered by the polysaccharide moiety. The linkage of a polysaccharide of a size smaller than glycogen, as in PG-200, has no effect on the binding of the sugar nucleotide derivative to glycogenin but diminishes its transglucosylation activity increasing the activation energy for the reaction.

MATERIALS AND METHODS

Materials. [β-^{32}P]5-azidouridine 5′-diphosphoglucose (14.7 mCi/μmol) was purchased from Research Products International Corp. (Illinois). UDP-[^{14}C]glucose (300 μCi/μmol) was purchased from Instituto de Investigaciones Bioquímicas Fundación Campomar (Buenos Aires, Argentina). Oyster glycogen, leupeptin, pepstatin, APMSF, Triton X-100, Q Sepharose (fast flow), UDP-glucose, Mes, DBM, trypsin and trypsin inhibitor were from Sigma (St. Louis, MO). α-Amylase from *Bacillus subtilis* was from Boehringer Mannheim and C_{18} cartridges from Waters (Milford, MA).

Proteoglycogen and glycogenin. Proteoglycogen having polysaccharide-bound glycogenin as the only protein constituent was prepared from rabbit skeletal muscle as described previously (5). Glycogenin was released from proteoglycogen (150 μg in glycogenin) by amylolysis with 3 μg of α-amylase (purified as indicated before (12)) for 5 h at 25°C, in 1.0 ml of 3 mM Tris–acetate, pH 7.5, containing 0.06% sodium azide and 0.03 mM each of leupeptin, pepstatin and APMSF. This treatment results in total digestion of the bound glycogen. As reported previously (12), α-amylase and the reaction products of glycogenolysis present in the amylolyzed sample of proteoglycogen do not interfere with the transglucosylation assay described below.

Preparation of PG-200. For the preparation of PG-200, the polysaccharide moiety of proteoglycogen was partially digested for 2 h with α-amylase as indicated above and amylolysis stopped by the addition of 50 μM acarbose. The amylolyzed mixture (up to 0.85 ml) was cooled in an ice-water bath, made 60 mM in NaCl and immediately passed, in the cold room, through a small column (0.2 ml) of Q Sepharose equilibrated with 60 mM NaCl in 20 mM Tris-acetate buffer, pH 7.5, containing 50 μM acarbose (buffer/acarbose). The column was washed with 1.8 ml of this solution, followed by 0.4 ml of buffer/acarbose containing 100 mM NaCl and PG-200 was eluted with 0.4 ml of buffer/acarbose containing 125 mM NaCl (Fig. 3a).

Digestion with trypsin. Proteoglycogen (1.0 μg in glycogenin) was incubated with trypsin (0.03%) for 2 h at 4°C, followed by the addition of trypsin inhibitor (0.09%) and digestion of its polysaccharide moiety with α-amylase (0.1 μg) for 4 h at 25°C.

Assay for transglucosylation activity. The incubation mixture contained, in a final volume of 15 μl: 8 μM UDP-[^{14}C]glucose, 0.1 M Mes pH 7.0, 5 mM $MnSO_4$, 0.2 mM DBM and 1.0 μg (in glycogenin) of intact or amylolyzed proteoglycogen, or PG-200. The incubation was done at 30°C for 7 min, and the reaction was terminated by addition of 4 μl of 0.1 M EDTA, the solution was made 1 mM in glucose and 2 mM in UDP-glucose in a final volume of 200 μl and passed through a C_{18} cartridge. The cartridge was washed with 3.0 ml of water and the [^{14}C]glucosylated DBM (DBMT) eluted with 0.6 ml of methanol and counted after addition of scintillation solution. The labeled DBMT eluted with methanol was identified by TLC as described before (13).

Photolabeling reactions. Photolabeling of intact or amylolyzed proteoglycogen (1.0 μg in glycogenin) was conducted in Eppendorf tube containing 50 mM Tris–HCl buffer, pH 7.5, 5 mM $MnSO_4$ and 10 μM [β-^{32}P]5N_3UDP-Glc (total vol 15 μl). The mixture was incubated for 20 s at 2°C followed by UV irradiation with a hand-held 254-nm UV lamp for 3 min. After the glycogenin moiety of proteoglycogen was released by amylolysis, the samples were subjected to SDS-PAGE and autoradiography as indicated before (14). For competition studies, the sample was incubated with UDP-glucose (100 μM) for 1 min at 2°C in 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $MnSO_4$, 10 μM [β-^{32}P]5N_3UDP-Glc was added and the reaction mixture was incubated for 20 s, irradiated and analysed as indicated.

Quenching by acrylamide. Fluorescence quenching studies were carried out on an Aminco SLM 4800C spectrofluorometer by adding aliquots (2 μl) of freshly prepared 7.6 M acrylamide to 1.0 ml of the intact or amylolyzed proteoglycogen solutions (150 μg in glycogenin) in 3 mM Tris–acetate, pH 7.5, containing 0.06% sodium azide and 0.03 mM each of leupeptin, pepstatin and APMSF. The samples were excited at 295 nm and the fluorescence emission measured at 340 nm. Fluorescence lifetimes were measured in the same spectrofluorometer using the frequency domain mode.

UV absorption spectra. Protein absorption spectra were taken in a Shimadzu UV-PC 1600 spectrophotometer equipped with a Peltier control of temperature using a 1 cm path cell. Fourth-derivative spectra were taken using the software provided with the spectrophotometer. Samples of the intact and amylolyzed proteoglycogen solutions (150 μg/ml in glycogenin) contained 3 mM Tris-acetate pH 7.5, 0.06% sodium azide and 0.03 mM each of leupeptin, pepstatin and APMSF. The absorbance of a same sample was scanned from 230 to 350 nm after successive 8-min heating at the desired temperatures. Light scattering due to glycogen was corrected with a solution containing an equivalent amount of protein-free glycogen from oyster in the reference cell. To study the reversibility to heat denaturation different samples were heated at the indicated temperatures for 8 min, rapidly cooled at 2°C and allowed to equilibrate at 30°C before taking the absorption spectra or measuring the transglucosylating activity. The activity recovered from the heated glycogen-bound glycogenin was measured after the enzyme was released by amylolysis as indicated above.

Arrhenius plot. Arrhenius plot for the rate of transglucosylation of glycogenin and PG-200 was carried out measuring the activity as indicated above, after 10-min incubations at 18, 21, 24, 27, and 30°C.

Other methods. SDS–PAGE was carried out as described (4) on 10% (w/v) acrylamide resolving gels with a 3% stacking gel.

RESULTS AND DISCUSSION

Effect of Linked Glycogen on the Transglucosylation Activity of Glycogenin and Binding of UDP-Glucose

It has been described that the autoglucosylating activity of glycogenin ceases after the incorporation of

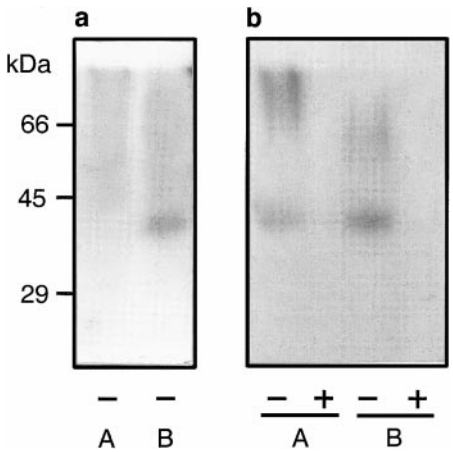

FIG. 1. UDPG-binding to proteoglycogen glycogenin. Intact (A) and amylolyzed (B) proteoglycogen samples were incubated for 1 min at 2°C with (+) or without (−) the addition of 100 μM UDPG and photolabeled with [β-^{32}P]5N$_3$UDP-Glc in the absence (a) or presence (b) of 33% DMSO. After photolabeling, the glycogenin moiety of the intact proteoglycogen samples was released by amylolysis and the samples were subjected to SDS–PAGE and autoradiography (see Materials and Methods).

eight to eleven glucose molecules (7, 15), whereas transglucosylation of exogenous substrates occurs even when the tyrosine residue of the enzyme is occupied by the autosynthesized oligoglucan (5). Glycogen linked to glycogenin as in proteoglycogen, totally abolishes the transglucosylation activity of the enzyme (5), while unbound glycogen had no effect (results not shown). The absence of transglucosylation activity in proteoglycogen could be due to the inaccessibility of substrates to its glycogenin moiety. As reported before (14), we labeled the uridine binding site of the UDP-glucose-binding domain of glycogen-free glycogenin using the photoprobe [β-^{32}P]5-azido-UDP-glucose. No photoinsertion of the azidouridine derivative was observed when proteoglycogen was subjected to photolabeling (Fig. 1a). Thus, the absence of transglucosylation activity in proteoglycogen would be due to inaccessibility of the sugar nucleotide to its glycogenin moiety. The results obtained with acrylamide and trypsin (see below) indicate that it is not the inaccessibility of substrates to the enzyme the cause of glycogenin inactivation by linked glycogen.

Accessibility of Acrylamide and Trypsin to Glycogen-Bound Glycogenin

To study the accessibility of substrates to the glycogenin moiety of proteoglycogen, the quenching of the fluorescence of tryptophan residues by acrylamide was measured (Fig. 2). We have obtained the same values of the Stern–Volmer constant, K_{SV} = 7.2 M^{-1}, for the fluorescence quenching of polysaccharide-free and -bound glycogenin. K_{SV} is the product between the collisional rate constant and the fluorescence lifetime (16). A fluorescence lifetime of 5 ns was measured for both, proteoglycogen and glycogenin, which results in a collisional rate constant of 1.44×10^9 M^{-1} s^{-1}, a value within the diffusional limit in solution (17). Trypsin was also accessible to proteoglycogen glycogenin. This was deduced from the absence of transglucosylating activity in an amylolyzed sample of proteoglycogen that had been previously subjected to digestion with trypsin, followed by neutralisation of the protease activity with trypsin inhibitor and amylolysis of the polysaccharide moiety with α-amylase (result not shown). These results indicate that glycogen does not hinder the diffusion of a solute as small or large as acrylamide or trypsin, respectively.

Restoration of the UDP-Glucose Binding to Proteoglycogen Glycogenin

It is known that hydrogen bonds between the glucose monomers stabilize the α-helix structure of the glycogen chains (18). Hydrogen bonds might be also occurring between the polyhydroxylated chains of glycogen and amino-acid residues of the protein, thus affecting the transglucosylation activity. To ascertain whether the binding of UDP-glucose was influenced by such a type of bonds, the photolabeling with [β-^{32}P]5-azido-UDP-glucose was carried out in the presence of DMSO. The chaotropic agent was used at a concentration that disrupts the hydrogen bonds stabilising the α-helical structure of α-1,4-glucan, as judged from the bleaching caused in the blue color of soluble starch-iodine/iodide complex. In the presence of 33% DMSO proteoglycogen was photolabeled with the azidouridine derivative (Fig. 1b). The radioactivity observed in the upper zone of the running gel might result from an incomplete amylolysis of the polysaccharide moiety of the labeled proteo-

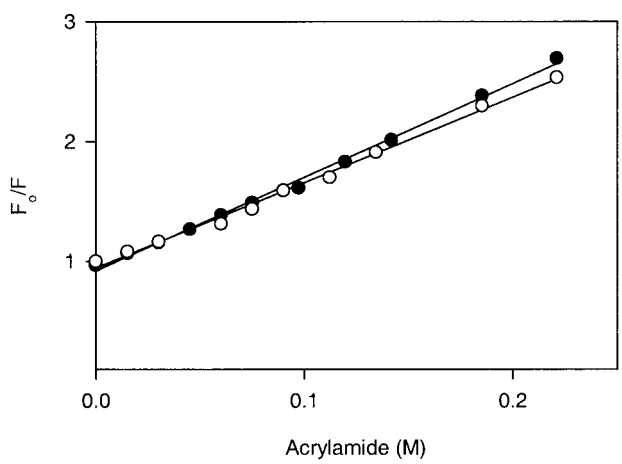

FIG. 2. Accessibility of the protein moiety of proteoglycogen to acrylamide. The quenching by acrylamide of the intrinsic tryptophan fluorescence of the glycogen-free (●) and glycogen-bound (○) enzyme was carried out as indicated under Materials and Methods.

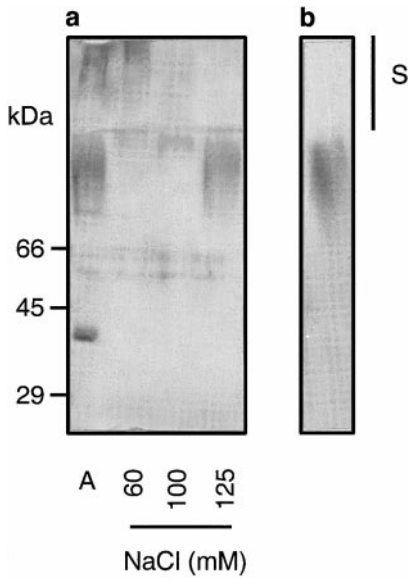

FIG. 3. Isolation and UDPG-binding activity of PG-200. (a) Proteoglycogen was amylolyzed and passed through a column of Q Sepharose and PG-200 was eluted as indicated under Materials and Methods. Samples of the amylolyzed mixture before chromatography (A) and of fractions eluted with 60, 100, and 125 mM NaCl were subjected to SDS–PAGE and silver stain. (b) PG-200 was photolabeled and subjected to SDS–PAGE and autoradiography as indicated in Fig. 1. S, indicates the zone corresponding to the stacking gel.

glycogen, caused by the presence of DMSO. The preincubation with UDP-glucose prior to photolabeling inhibited the binding of the azido derivative to both proteoglycogen glycogenin and the polysaccharide free enzyme, thus proving the specificity of the binding.

No transglucosylating activity was detectable in proteoglycogen assayed in 33% DMSO, in spite of the observed restoration of the binding of sugar nucleotide to its glycogenin moiety and the activity shown in DMSO by glycogen-free glycogenin (30% of the activity measured in the absence of DMSO; result not shown). Thus, the transglucosylation inactivity of proteoglycogen is not solely due to the inability to bind UDP-glucose, in which hydrogen bonds seem to be involved. Linked glycogen might also be causing a distortion of the catalytic site of the enzyme. The studies carried out with PG-200 (see below) are consistent with this assumption.

Recovery of Transglucosylating Activity of Proteoglycogen by Shortening the Size of the Linked Polysaccharide

Mild digestion of proteoglycogen with α-amylase followed by passage through a Q Sepharose column allowed the isolation of PG-200, a proteoglycogen species having a polysaccharide moiety of lower size than native glycogen (Fig. 3a). PG-200 was photolabeled with [β-^{32}P]5-azido-UDP-glucose (Fig. 3b). However, it was 60% less active for transglucosylation than the polysaccharide-free enzyme. In order to determine whether the linked polysaccharide affected the energetics of the catalytic process, the activation energy of the transglucosylation reactions were compared measuring the temperature dependency of the initial rate of DBM glucosylation by PG-200 and glycogenin. Figure 4 shows that the temperature dependencies were different, indicating that the catalytic process is altered by the bound polysaccharide moiety increasing the energy barrier for glucosylation.

Effect of Linked Glycogen on the Thermal Stability of Glycogenin

To study the thermal stability of the glycogen-free and -bound glycogenin the fourth derivative of the UV absorption spectra were taken at different temperatures (Fig. 5a). Several parameters of the fourth-derivative spectra can be used to study protein unfolding (19). Here we show the position of the peak at around 292 nm. A blue shift of this peak can be interpreted as the transfers of tryptophan residues to a more polar environment caused by the protein unfold. Heating glycogen-free and glycogen-bound glycogenin resulted in a small and reproducible blue shift. The mean point for the transitions were 51 and 64°C, respectively, which indicate that linked polysaccharide increases the thermal stability of the enzyme.

In order to analyse the reversibility of the changes caused by heat treatment, samples of glycogen-free and

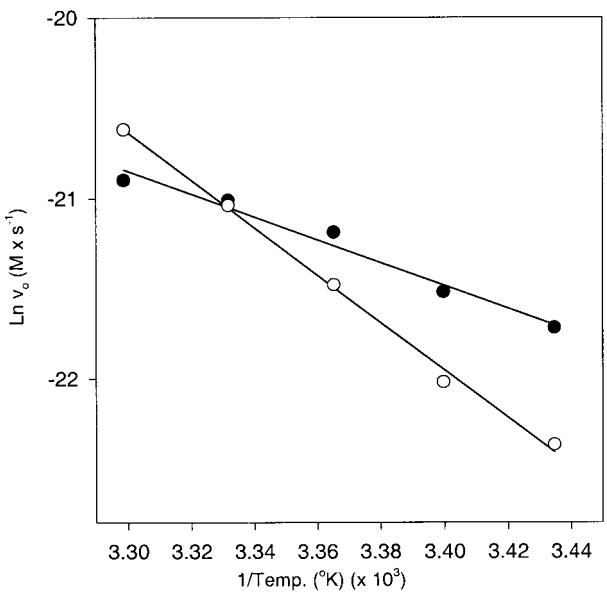

FIG. 4. Arrhenius plot. The steady-state transglucosylation activity of glycogen-free glycogenin (1.0 μg) (●) and PG-200 (2.5 μg in glycogenin) (○) was measured at the indicated temperatures (see Materials and Methods).

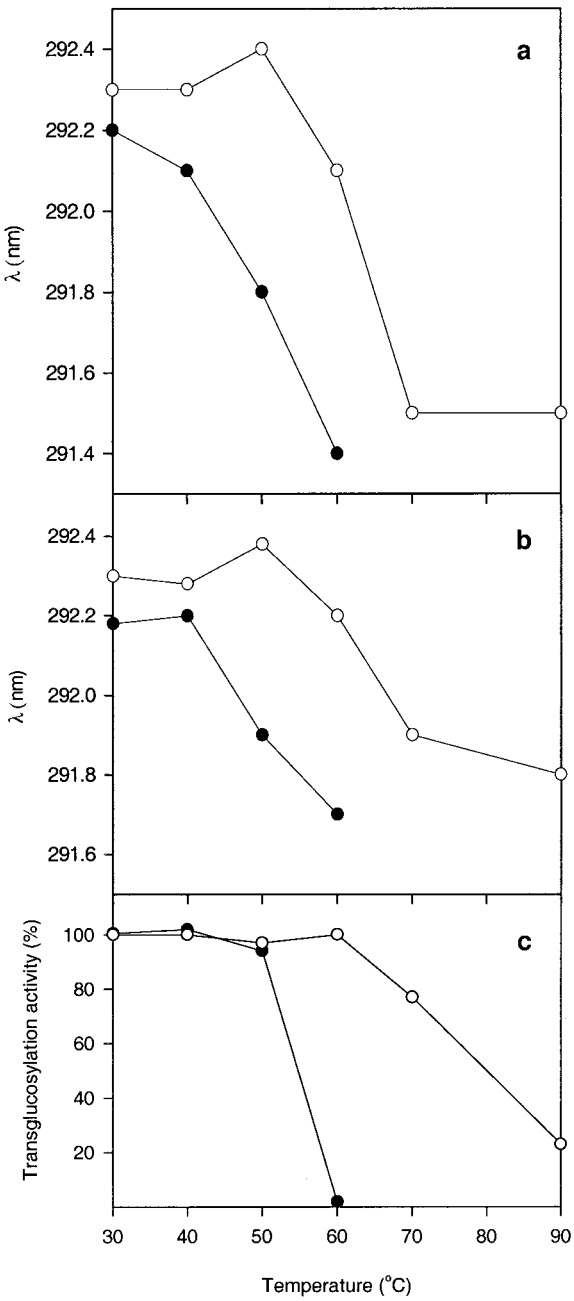

FIG. 5. Effect of linked glycogen on the thermal stability of glycogenin. The fourth-derivative spectrum (a, b) and transglucosylation activity (c) of glycogen-free (●) and glycogen-bound (○) glycogenin heated at the indicated temperatures. The spectra were taken and the activity was measured immediately after heating (a) or after cooling at 2°C and equilibrating to 30°C (b, c). For details see Materials and Methods.

glycogen-bound enzyme were heated at the indicated temperatures, rapidly cooled at 2°C and equilibrated at 30°C before the UV spectrum was taken (Fig. 5b) and the transglucosylating activity measured (Fig. 5c). Glycogen-free glycogenin subjected to 50°C showed, after cooling, a blue shift of 0.3 nm and a recovery of 100% of the transglucosylating activity. A blue shift of 0.4 nm, with a recovery of 80% of the glycogenin activity, was observed for the glycogenin released from proteoglycogen subjected to 70°C. When glycogen-free glycogenin was subjected to 60°C and proteoglycogen to 90°C, a blue shift of 0.5 nm occurred (Fig. 5b), with no or little (20%) recovery of activity, respectively (Fig. 5c). Thus, a blue shift of 0.5 nm would correspond to a distortion of the glycogenin conformation enough to produce inactivation. The increased stability of glycogenin under the proteoglycogen form might result from hydrogen bonds between the polyhydroxylated hydrocarbon chains and aminoacid residues that both, increase the unfolding temperature and help to refold to the native conformation.

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