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Factors affecting oligomerization status of UDP-glucose pyrophosphorylase

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Abstract

UDP-glucose pyrophosphorylase (UGPase) is involved in the production of UDP-glucose, a key precursor to polysaccharide synthesis in all organisms. UGPase activity has recently been proposed to be regulated by oligomerization, with monomer as the active species. In the present study, we investigated factors affecting oligomerization status of the enzyme, using purified recombinant barley UGPase. Incubation of wild-type (wt) UGPase with phosphate or Tris buffers promoted oligomerization, whereas Mops and Hepes completely dissociated the oligomers to monomers (the active form). Similar buffer effects were observed for KK127-128LL and C99S mutants of UGPase; however, the buffers had a relatively small effect on the oligomerization status of the LIV135-137NIN mutant, impaired in deoligomerization ability and showing only 6–9% activity of the wt. Buffer composition had no effect on UGPase activity at UGPase protein concentrations below ca. 20 ng/ml. However, at higher protein concentration the activity in Tris, but not Mops nor Hepes, underestimated the amount of the enzyme. The data suggest that oligomerization status of UGPase can be controlled by subtle changes in an immediate environment (buffers) and by protein dilution. The evidence is discussed in relation to our recent model of UGPase structure/function, and with respect to earlier reports on the oligomeric integrity/activity of UGPases from eukaryotic tissues.

Keywords: Cellulose; Protein oligomerization; Sucrose; UDP-glucose synthesis

1. Introduction

Oligomerization is one of the key regulatory processes that affect protein function/activity (Torshin, 1999; Peneff et al., 2001; Wilczynska et al., 2003; Perugini et al., 2005). A number of enzymes have active monomeric and inactive oligomeric forms (or vice versa), which underlies the importance of interglobular interaction(s). Although the oligomerization phenomenon is widely studied in vitro as part of folding process, the physiological mechanisms and biological roles of the oligomerization are not clearly understood.

Recently we have shown that barley UDP-glucose pyrophosphorylase (UGPase) [EC 2.7.7.9], a major activity producing UDP-glucose (UDPG), exists as a mixture of monomers, dimers and higher order oligomers, with the monomer as by far the most active species (Martz et al., 2002). The oligomerization phenomenon was demonstrated both by molecular sieving chromatography and by native PAGE. The oligomerization of UGPase may play a regulatory role and, thus, be of importance for any process requiring UDPG as substrate. In plants, UDPG has an essential function as a direct or indirect precursor of

Abbreviations: aa, amino acids; *AGX, UDP-N*-acetylglucosamine pyrophosphorylase; DTT, dithiothreitol; I-loop, insertion loop; PAGE, polyacrylamide gel electrophoresis; PPi, pyrophosphate; UGPase, UDP-glucose pyrophosphorylase.

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sucrose and cell wall components (e.g. cellulose, hemicelluloses) (Witt, 1992; Kleczkowski, 1994a; Amor et al., 1995; Johansson et al., 2002), and is utilized in the synthesis of carbohydrate moiety of glycolipids, glycoproteins and a variety of secondary metabolites, among other functions (Flores-Díaz et al., 1997; Bishop et al., 2002). Besides oligomerization, other possible posttranslational means of regulation for UGPase include phosphorylation (Rutter et al., 2002) and *O*-glycosylation (Wells et al., 2003), whereas factors that affect UGPase activity via (post)transcriptional regulation include phosphate deficiency stress, cold, light exposure, sucrose feeding, salinity and cadmium excess (Ciereszko et al., 2001a,b, 2005; Goulard et al., 2001; Repetto et al., 2003; Kleczkowski et al., 2004; Ciereszko and Kleczkowski, 2005).

We have earlier demonstrated that a highly oligomerized mutant of barley UGPase (LIV135-137NIN), impaired in deoligomerization, had very little catalytic activity (Martz et al., 2002). This suggested that the ability to depolymerize may be crucial for catalytic efficiency of the enzyme. The mutation lies close to the so called insertion loop (I-loop), which is responsible for "locking up" the UGPase in a dimeric conformation (Geisler et al., 2004). The I-loop, located at the *C*-terminus, is a characteristic of UGPases from both plant and animal tissues (Geisler et al., 2004).

In the present study, we demonstrate that: (i) oligomerization of wt UGPase, but not the NIN mutant, is fully reversible, depending on conditions, (ii) that certain buffers have opposite effects on structural integrity of UGPase, and (iii) that dilution likely promotes deoligomerization of the protein, regardless of the buffers used, leading to the formation of fully active monomers. The data suggest that oligomerization status of the enzyme can be controlled by subtle changes in hydrophobicity of the environment and by molecular crowding conditions.

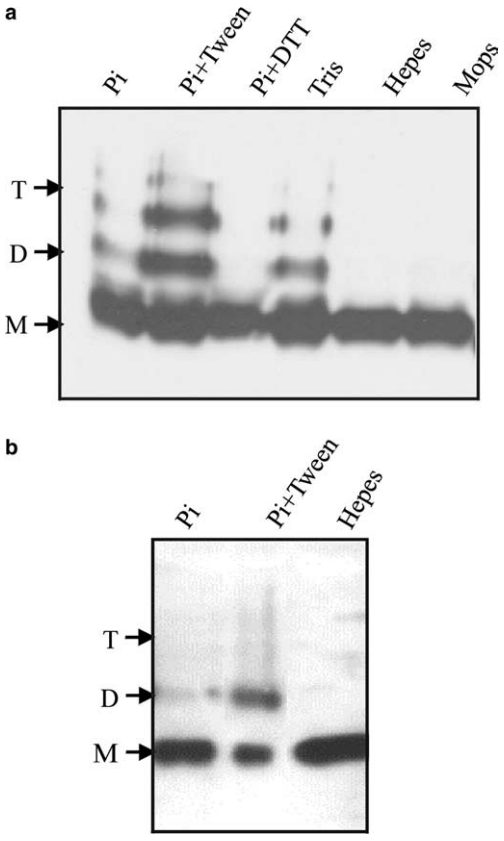

Fig. 1. Oligomerization of barley UGPase. Western blot analysis after native PAGE of (a) purified recombinant barley UGPase pretreated in several incubation conditions: Pi (phosphate elution buffer) in the absence or presence of 0.05% Tween-20 or 5 mM DTT; or in 0.1 M Tris (pH 7.6); 0.1 M Hepes (pH 7.6); and 0.1 M Mops (pH 7.6); and of (b) UGPase from crude leaf extracts in Pi (phosphate extraction buffer) in the absence or presence of 0.05% Tween-20; or 0.1 M Hepes (pH 8.0). Protein aliquots of 1.2 μg (a) and 60 μg (b) were loaded per each lane. Positions of monomers (M), dimers (D) and tetramers (T) are indicated with arrows.

2. Results and discussion

2.1. UGPase can oligomerize and deoligomerize, depending on buffer composition

Purified wild-type (wt) barley UGPase was incubated in different buffers and run on native PAGE, followed by western-blotting. In the presence of phosphate buffer or Tris, UGPase appeared on the gel as several oligomers of different sizes (Fig. 1(a)). The oligomerization status depended on incubation conditions. Addition of Tween-20 (but not Triton X-100, data not shown), promoted appearance of higher order oligomers, whereas 5 mM dithiothreitol (DTT) promoted deoligomerization, and Hepes or Mops completely dissociated all the oligomers (Fig. 1(b)). Addition of β-mercaptoethanol (10 mM) had a similar effect as DTT (data not shown). Quantitative analysis of band intensities on ECL films revealed that, for native enzyme pretreated with phosphate, the monomeric form comprised over half of UGPase protein whereas, in the presence of Tween-20, monomers, dimers and tetramers were in about equal amounts. The same oligomerization pattern was observed for the purified UGPase before and after desalting (Sephadex G-25 column) into 0.1 M sodium phosphate (pH 8.0) (data not shown), ruling out any effect of salt or imidazole, present in the elution buffer, on the polymerization process.

The results obtained for purified recombinant UGPase were confirmed for the enzyme from crude leaf preparations when subjected to native PAGE, followed by specific detection of the UGPase protein by western-blotting. Whereas higher order polymers were not as prominent for crude leaf UGPase as for recombinant enzyme, a dimer was detected in Tris, and its amount increased in the presence of Tween-20, while only monomers were found in Hepes (Fig. 1(b)). This indicated that oligomerization of the purified enzyme is not an artefact of the heterologous expression and purification procedures.

The different effects of zwitterionic (Hepes and Mops) as well as Tris and phosphate buffers on oligomeric status of UGPase must lie in the chemical structure of the buffers. We do not know whether it is the slightly higher

hydrophobicity of the zwitterionic buffers, compared to Tris (or phosphate buffer), or their sulfonate and heterocyclic aliphatic groups that are responsible for deoligomerization of UGPase. For instance, Hepes was earlier found to be specifically bound to several crystallized proteins, including SET domain protein methyltransferase and glutathione-*S*-transferase (Ji et al., 1997; Trievel et al., 2002). Hepes–protein interactions were stabilized both by hydrophobic interactions and interactions with positively charged side chains. However, regardless of the exact mechanism of buffer effects on the oligomerization status of UGPase, it is clear that the energy barrier between the monomeric and oligomeric states of the enzyme is rather low. This rises the possibility that the monomers–oligomers balance of UGPase may be easily affected in vivo.

The oligomerizing action of Tween-20 (Fig. 1) possibly relates to its frequently observed stabilizing effects on proteins (Lewinsohn et al., 1992; Klinz, 1994), but the exact mechanism with respect to its effect on UGPase is unknown at present. The deoligomerizing effect of DTT on UGPase (Fig. 1(a)) may possibly result from reduction of internal disulfide bridge(s) in UGPase subunit, bringing about changes in conformation that lead to deoligomerization. The DTT may have also acted as a reductant of possible disulfide bridge(s) between the oligomers. In the latter case, however, the existence of such bridge(s) was ruled out for the UGPase, based on two observations: the reversibility of the oligomerization by Hepes and Mops in the absence of any reducing reagent (Fig. 1), and the presence of a single band on SDS–PAGE gels (in nonreducing conditions) (data not shown) (Martz et al., 2002).

We have also tested the possibility that substrates/products of UGPase could promote the deoligomerization. However, neither incubation of the enzyme with glucose-1-P and/or UTP (substrates of the forward reaction of UGPase) or with UDPG and/or pyrophosphate (substrates in the reverse reaction) had any marked effect on oligomerization patterns on native PAGE gels (data not shown).

2.2. Oligomerization status of mutated UGPase

To further characterize the oligomerization process of UGPase, three site-directed mutants of UGPase were tested for their oligomerization status following incubation in different buffers (Fig. 2). The LIV (aa # 117–119) to NIN, and KK (aa # 127–128) to LL mutations were engineered in a conserved hydrophobic domain of UGPase (aa # 117–138) to affect possible protein–protein interactions (Martz et al., 2002), whereas C99S mutation affected substrate binding to the enzyme (Chang et al., 1996; Martz et al., 2002). Based on homology modeling of UGPase structure (Geisler et al., 2004), the NIN mutation is localized in a hydrophobic helix of the central (catalytic) domain, close to the I-loop that is involved in dimerization of UGPase monomers. The same model predicts location of the LL mutant at a surface-exposed loop of the central domain which links two conservative hydrophobic fragments: one fragment is in close proximity to the I-loop and the other is part of reactive center pocket. The C99S mutation is located at the so called "nucleotide-binding" loop which is part of the active site at the central domain (Geisler et al., 2004).

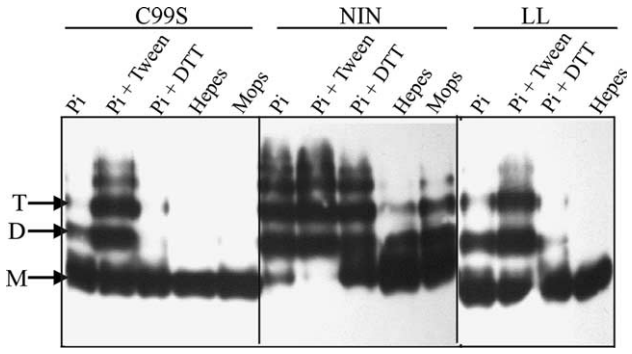

Fig. 2. Oligomerization of mutated UGPase. Western blot analysis after native PAGE of purified C99S, NIN and LL mutants of UGPase (2 μg each) that were pretreated in several incubation conditions: Pi (phosphate elution buffer) ±0.05% Tween-20 or 5 mM DTT; 0.1 M Hepes (pH 7.6); or 0.1 M Mops (pH 7.6). Positions of monomers (M), dimers (D) and tetramers (T) are indicated with arrows.

Oligomerization abilities of the mutant proteins were tested in the same conditions as the wt UGPase in Fig. 1. Most importantly, a high oligomerization capacity was observed for the NIN mutant, with very little monomer detected in the presence of Tween-20 and a substantial oligomerization even in Hepes or Mops buffer (Fig. 2). The C99S and LL mutants behaved similarly to wt, regardless of conditions (Fig. 2). However, the exchange of two Lys to two Leu in the LL mutant led to a modification in the net charge and a decrease in the pI (4.93 compared to 5.07 for the wt), reflected by a higher protein mobility during native PAGE.

2.3. Oligomerization status and activity of UGPase

Since the oligomerization state of UGPase may change depending on buffer composition (Fig. 1), the same buffers were used in assays to measure activities of wt, C99S, NIN and LL mutants (Table 1). Surprisingly, the wt UGPase

Table 1
Activities of wt and mutants of UGPase, assayed in different buffers

Assay buffer (100 mM, pH 7.6)	Activity (%)			
	wt	NIN	C99S	LL
Tris	100	8	34	48
+Tween-20	92	9	34	45
+DTT	126	11	44	57
Mops	97	6	58	47
Hepes	92	7	50	38
Phosphate	51	6	19	27

Activities were measured in the presence of saturating or near-saturating concentrations of both substrates (1 mM PPi and 1 mM UDP-glucose) and using 6–30 ng of purified UGPase per assay (values shown were standardized for the same amount of UGPase, and are the mean of three assays; the standard deviation was less than 10%).

had similar activities in Tris, Hepes or Mops buffers. Thus, under our assay conditions, catalysis of UGPase was not affected by oligomerization-promoting Tris, nor deoligomerization-promoting Hepes and Mops buffers. Only half of the activity was measured in 100 mM sodium phosphate, but this was most probably due to a weak inhibitory effect of phosphate on UGPase activity, as earlier observed for barley malt UGPase (Elling, 1996). Thus, although phosphate (at a micromolar Ki) is an important regulatory metabolite during sucrose and starch conversions (Edwards and Walker, 1983; Kleczkowski, 1994b, 1999), it is rather unlikely to have any significant regulatory role for UGPase under physiological conditions. Addition of 0.05% Tween-20 had no significant effect on UGPase activity, whereas 5 mM DTT increased the activity by about 25% (Table 1); the latter may have acted by influencing redox status of cysteines in the UGPase monomer.

Compared to the wt UGPase, the mutant proteins displayed reduced activities in all buffers tested, with e.g. 8%, 34%, and 48% of the wt activity in Tris for the NIN, C99S, and LL proteins, respectively (Table 1). Generally, activities of the mutants followed the same variation as the wt UGPase, depending on buffer or the presence of DTT or Tween-20. However, a different behaviour was observed for C99S which had a higher activity in Hepes and Mops compared to that in Tris. The NIN mutant had the lowest activity, regardless of the buffers used (Table 1). The low activity of the NIN mutant in all buffers coincides with its highly oligomerized status and with a relatively small amount of the monomer formed (Fig. 2). In our earlier study, staining activity assays for UGPase following native PAGE have unequivocally identified the monomer as by far the main active form of the enzyme (Martz et al., 2002).

Since both C99S and LL mutations had no effect on oligomerization of UGPase (Fig. 2), their low enzymatic activities probably result entirely from effects on catalysis/substrate binding of the modified protein. The C99S has been earlier demonstrated to affect binding of PPi (Chang et al., 1996; Martz et al., 2002). On the other hand, the LL is located at protein surface in central (catalysis/substrate binding) domain and it has been predicted that the mutation (replacing two Lys for two Leu residues) will result in conformational change in this region (Geisler et al., 2004). It appears, thus, that this change was sufficient to affect the activity of the modified protein (Table 1).

2.4. Dilution as deoligomerizing factor

An intriguing aspect of the present studies was that UGPase had the same or similar activity both under conditions promoting oligomerization (Tris, Tween-20) and those resulting in deoligomerization (Mops, Hepes) (Table 1), even though monomer was earlier demonstrated as by far the most active form of UGPase (Martz et al., 2002). One possibility is that dilution of the enzyme in assays leads to dissociation of oligomers to monomers, regardless of buffers used. Dilution is recognized as a potent deoligomerizing factor for a number of proteins (e.g. Dudenhöffer et al., 1998). We have addressed this issue by testing the effect of dilution of the enzyme on its activity in assays containing Tris, Hepes and Mops buffers (Fig. 3). At assay concentrations od UGPase lower than ca. 20 ng/ml, the activity in all buffers tested was similar and proportional to the amount of the enzyme. However, at concentrations of UGPase protein higher than 20 ng/ml, the activity in Tris (but not in Mops and Hepes) underestimated the amount of UGPase protein. It appears thus that at low UGPase concentration the enzyme most likely exists in its most active state (monomer), regardless of buffers used, whereas as UGPase concentration increases the oligomerizing effect of Tris is apparent, resulting in loss of correlation between UGPase assay content and activity. This also suggests that the oligomerization is under equilibrium that is rapid enough to account for similar initial activities of the diluted (<20 ng/ml) enzyme, both in oligomerization-promoting (Tris) and deoligomerization-promoting (Mops, Hepes) media. When the equilibrium is affected, as in NIN mutant (Fig. 2), the catalytic efficiency is greatly reduced (Table 1).

It should be emphasized that in native PAGE/immunoblot studies that directly assessed the oligomerization status of UGPase (Figs. 1 and 2), we loaded 1.2–2 μg of purified UGPase, which is two or three orders of magnitude more than was used in activity assays (4–68 ng in Fig. 3, and 6–30 ng in Table 1). Using native PAGE/immunoblot approach we were unable to reliably quantify protein bands when 50 ng or less of purified UGPase were loaded on the gel (data not shown). Thus, we were limited by sensitivity of our immunodetection system to see effects of dilution on oligomerization states of UGPase.

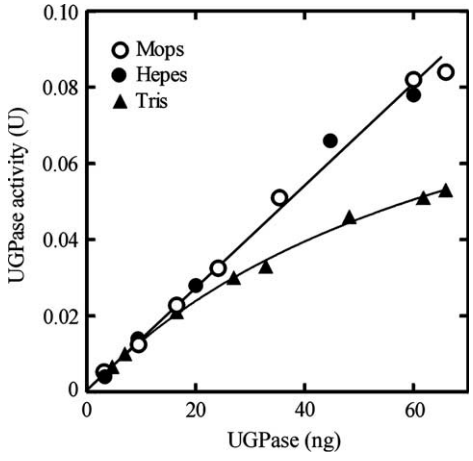

Fig. 3. The relationship between UGPase amount and activity in assays containing either Tris, Mops or Hepes buffers (at 100 mM each, pH 7.6). Prior to assays the enzyme was diluted 50-fold into the respective buffer (to 2.8 μg/ml) and kept on ice overnight. UGPase was added to assays at a range of 4–68 ng (per 1 ml assay mixture).

2.5. Oligomerization as regulatory mechanism

Previous detailed kinetic data on purified UGPases from human liver and potato tubers have suggested a possibility of the existence of two different active forms of UGPase, consistent with different conformational and/or oligomerization states (Sowokinos et al., 1993; Duggleby et al., 1996). Interestingly, in oligomeric human UDP-*N*-acetylglucosamine pyrophosphorylase (AGX), which is structurally related to UGPase (Peneff et al., 2001; Kleczkowski et al., 2004; Geisler et al., 2004), monomer is also by far the most active form of the enzyme; a dimer of AGX was proposed to dissociate to monomers under assay conditions (Peneff et al., 2001). For both UGPase and AGX, the dimerization process apparently modifies the structural environment of the active site, which is open in the monomer and occluded at the dimer interface.

In Fig. 4, we have summarized the effects of different experimental conditions on oligomerization status of UGPase. Both the nature of buffers and the degree of dilution of UGPase are key factors involved. In this respect, it is worth mentioning that in the cytosol, where most of UGPase is localized (Kimura et al., 1992; Kleczkowski, 1994a; Mikami et al., 2001), total protein concentration was estimated as 50–400 mg/ml (Minton, 2001; Chebotareva et al., 2004; Young et al., 2004). This corresponds to at least 0.1–0.8 mg UGPase protein per ml, based on the abundance of this protein in plant tissues (Kleczkowski, 1994a), and would likely result in a preferentially oligomerized (less active) form of UGPase in vivo. However, in analogy to the effects of different buffers on UGPase oligomerization status (Fig. 1), subtle changes in hydrophobicity due, e.g. to interactions with other cytosolic proteins may overcome the protein concentration effect. This could tip the balance toward monomers, the active form of UGPase.

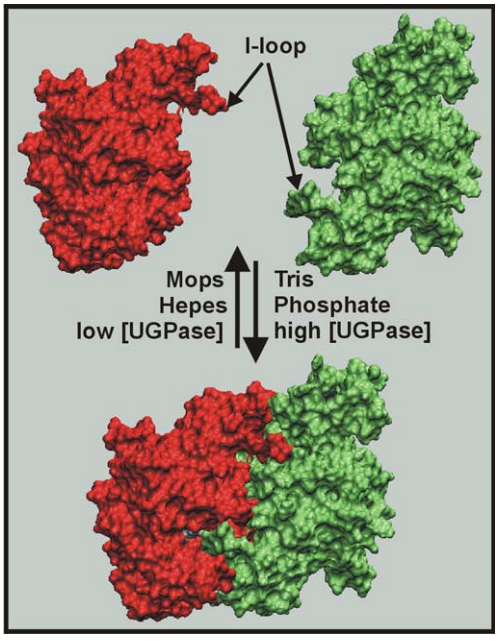

Fig. 4. A summary for effects of buffers and dilution on oligomerization status of UGPase. The I-loop, which was earlier proposed to stabilize UGPase dimer (Geisler et al., 2004), is marked by arrows.

Another aspect that has emerged from the present study is the strict dependence of the oligomerization status of UGPase on commonly used buffers and reagents. This was unexpected and, to our knowledge, has only seldom been reported for other proteins, e.g. for bacterial RecA protein (Brenner et al., 1990). The opposite effects of certain buffers on the structural integrity of barley UGPase may have implications for rationalization of results of earlier studies on UGPases from other sources. In fact, in the classical study reporting octameric structure of calf liver UGPase (Levine et al., 1969), the protein (prior to determination of its oligomeric status) was maintained in Tris, which in the present study induced oligomerization of the barley enzyme. It would be interesting to test systematically whether the oligomerization phenomenon characterized by low energy barrier between the monomers and dimers, and therefore easily reversible, is indeed a common regulatory mechanism for all UGPases.

3. Experimental

3.1. Purification of recombinant UGPase

Cloning of the full coding cDNA sequence of barley UGPase (GenBank accession # X91347) (Eimert et al., 1996), generation of site-directed mutants, and expression in *Escherichia coli* using pET23d expression vector (Novagen) were as previously described (Martz et al., 2002). Following cell lysis and centrifugation, the His-tagged protein was purified from the soluble fraction under native conditions on Ni-NTA spin columns according to the manufacturer's instructions (Quiagen). Bound protein was eluted from the column with elution buffer (0.1 M sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole) and 25% (final concentration) glycerol was added before storage at −20 °C. Protein content was quantitated according to Bradford method using the Bio-Rad Assay kit (Bio-Rad) and with BSA as protein standard.

3.2. PAGE and immunoblotting

SDS–PAGE was run on slab gels containing 10% acrylamide according to Laemmli (1970). Following electrophoresis, the gels were stained with Coomassie Blue. Native 8.5% polyacrylamide gels (1.5 mm thick) were run at 4 °C according to the same procedure, but SDS (and β-mercaptoethanol) was omitted from samples and buffers. For analyses of UGPase oligomerization under different buffer conditions, purified protein in elution buffer was diluted into a given buffer (3–5-fold dilution) while keeping the same protein concentration in all the samples of a given experiment. Following 15–30 min incubation at 4 °C, protein samples were loaded onto native polyacrylamide gels for PAGE analyses.

Following native PAGE, the resolved proteins were transferred to nitrocellulose membrane and the UGPase protein was detected with rabbit antibodies raised against potato tuber UGPase (0.6 μg/ml) (Sowokinos et al., 1993) followed by goat anti-rabbit IgG coupled to peroxidase (Amersham). Specific labelling was detected with the ECL kit (Amersham).

3.3. Enzyme assays

UGPase activity was routinely measured in 1 ml assays containing 100 mM Tris (pH 7.6), 1 mM UDP-glucose, 1 mM pyrophosphate (PPi), 0.8 mM NAD, 1 mM $MgCl_2$, 2 units of phosphoglucomutase (Boehringer), 5 units of glucose-6-P dehydrogenase (Boehringer), and 6–30 ng of purified protein. The change in optical density was monitored spectrophotometrically (340 nm) at 23 °C, and its linear portion was used to determine UGPase activity. To study the effects of assay buffers and UGPase dilution on activity (see Table 1 and Fig. 3), the enzyme activity was determined at 100 mM of either Tris, Mops, Hepes or phosphate buffers (all assays at pH 7.6). One unit of UGPase activity was defined as the amount of the enzyme required to reduce 1 μmol NAD per min.

3.4. Preparation of barley leaf extracts

Etiolated leaves from 7-day-old barley (*Hordeum vulgare*) plants were homogenized in 2 volumes of extraction buffer (0.1 M sodium phosphate, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA), the extract was centrifuged for 3 min at 10,000*g* (4 °C), the resulting supernatant was supplemented with glycerol to 25% final concentration and stored at −20 °C. Where indicated, aliquots of the supernatant were supplemented with 0.05% Tween-20 or 0.1 M Hepes, pH 8.0, and were analyzed by native PAGE followed by Western blot.

3.5. Other methods

Quantification of relative amounts of monomers, dimers and higher order oligomers, based on protein band intensities following immunoblots, was carried out using Molecular Analyst software, version 1.5 (Bio-Rad).

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