Activation by polyamines, polycations, and ruthenium red of the Ca^{2+}-dependent glucan synthase from soybean cells

Heinrich Kauss and Wolfgang Jeblick

Department of Biology, University of Kaiserslautern, Postfach 3049, D-6750 Kaiserslautern, FRG

Received 9 April 1985

The 1,3-β-D-glucan synthase in microsomal preparations from suspension-cultured soy bean cells requires Ca^{2+} for activity. In the absence of Ca^{2+} the enzyme can also be activated by poly-L-Lys, poly-L-Orn and ruthenium red. Under these conditions it is either not or only slightly inhibited by La^{3+} and shows increased affinity for UDP-glucose. Spermine, when present alone at 57 μM, effects relatively little activation but cooperates with 5 μM Ca^{2+} to greatly enhance the affinity of the enzyme towards UDP-glucose, suggesting that both types of activation may occur at the same enzyme

1,3- β-D-Glucan synthase activation *Ca^{2+}/spermine cooperation* *La^{3+} inhibition* *Basic polyamino acid* *Ruthenium red*

1. INTRODUCTION

Callose is essentially a 1,3-β-D-glucan which possibly also contains minor portions of 1,4-β- or other linkages. It is often deposited at special sites on the cell walls of higher plants when they are newly formed or altered during development. Rapid callose deposition also plays an important role in cell or tissue repair mechanisms operating after mechanical injury or in the first line of defense against pathogenic fungi and virus (see [1,2]).

The plasma membrane located 1,3-β-D-glucan synthase (EC 2.4.1.34) appears to be involved in callose deposition. The enzyme therefore may require effective regulation in order to control the processes mentioned above. It has been shown with microsome preparations from soybean cells that the enzyme is strictly dependent on Ca^{2+} in the micromolar range [1]. In soybean cells, rapid callose formation occurs after membrane perturbation with the natural polycation chitosan. This process occurs only when external Ca^{2+} is present, suggesting that the Ca^{2+}-dependence of the 1,3-β-D-glucan synthase is of physiological significance [2,3]. We here present data showing that polyamines and polycations cooperate with Ca^{2+}-activation of the enzyme.

2. MATERIALS AND METHODS

Origin and growth of suspension-cultured soybean cells were as described [1,2]. The cells (2 g) were homogenized [2] in 4 ml of 100 mM Tes/NaOH, pH 7.0, containing 1 mM DTT, the washed microsomes [2] were suspended in 10 ml of 50 mM Tes/NaOH, pH 7.0, containing 1 mM DTT. In some experiments (fig.1), the microsomes were freed from endogenous Ca^{2+} by slowly rolling them in a test-tube with about 1 ml of chelating resin (Na^+-form), followed by settling for about 1 min and decantation. Trypsinization (50 μg/ml) of the microsome suspension was performed for 1 min at 25°C in the presence of 0.02% (w/v) digitonin and was terminated by addition of 100 μg/ml soybean trypsin inhibitor [1].

The standard enzyme assay consisted of 50 μl of microsomes and 50 μl of 50 mM Tes/NaOH, pH 7.0, containing 20 mM cellobiose, 16% (w/v) glycerol and 0.04% (w/v) digitonin. Further substances were added in 20 μl water and the reaction started with 5 μl UDP-[^{14}C]glucose (0.8 mM

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00145793/85/$3 30

final concentration, 57000 cpm). Standard incubation was 10 min at 25°C. When the UDP-glucose concentration was lowered (figs 3 and 4), the labelled substrate was increased up to 160000 cpm and the reaction time lowered appropriately (2 or 0.5 min) to ensure linearity of glucan formation with time The reaction was stopped by heating the tube to 95°C and the polymer formed was determined [1]. The values were corrected from the zero-time controls.

$[Ca^{2+}]$ was measured using scaled-up assay mixtures without substrate and a Radiometer-Selectrode [1] calibrated against a standard solution diluted in 50 mM NaCl, freed of Ca^{2+} by passage through a chelating resin.

All substances not mentioned before [1,2] were bought from Sigma. Ruthenium red was of practical grade; given concentrations were corrected for the low purity of 45%.

3. RESULTS

In agreement with earlier observations [1] the Ca^{2+} dependence of the 1,3-β-D-glucan synthase is again evident from table 1. Trypsinization renders the enzyme independent of Ca^{2+}. Under these conditions, however, Mg^{2+} causes a more pronounced increase in enzyme activity Even if any endogenous Ca^{2+} is bound by EGTA the enzyme becomes active by the presence of poly-L-Orn and ruthenium red, the effect of these substances being, to some extent, additive to the effect of Ca^{2+} Activation by poly-L-Orn and ruthenium red is generally more pronounced when the microsomes were trypsinized (table 1). Out of the various substances investigated, poly-L-Orn and poly-L-Lys were the most effective (table 2). Within the polyamines the degree of activation increases with the number of amino groups present in the molecule.

The activating effect of Ca^{2+} is counteracted by low concentrations of La^{3+} (fig.1). In contrast, when poly-L-Orn is used as an activator there is no inhibition at all by La^{3+}, whereas with ruthenium red only a relatively slight inhibition is observed at elevated La^{3+} concentrations.

The affinity of the Ca^{2+}-activated 1,3-β-D-glucan synthase towards its substrate UDP-glucose is low (figs 2 and 3). On a Lineweaver-Burk plot the data reported in fig.2 for saturating concentrations of Ca^{2+} give a nearly straight line only above 50 μM, with an apparent K_M of 0 57 mM (average of 4 experiments). Below 50 μM the curve is sigmoidal (figs 2 and 3). If activation is brought about by relatively high concentrations of poly-L-Orn or ruthenium red without Ca^{2+}, the enzyme becomes considerably more active at low UDP-glucose concentrations (fig.2). Values for K_M could not be calculated for these conditions as no straight line was observed in inverse plots. Substrate saturation is apparently reached (fig.2) and this allows estimation of half-saturation at about 50 and 100 μM UDP-glucose for the enzyme activated by poly-L-Orn and ruthenium red, respectively.

The importance of the various activators becomes more evident when they are combined and the concentration of all compounds involved is lowered. The Ca^{2+} present endogenously in the assay mixture (about 5 μM) is sufficient for about half-maximal activity of the enzyme at 0.8 mM

Table 1

Effect of various activators on 1,3-β-D-glucan synthase in native and trypsinized microsomes

Assay conditions	1,3-β-D-Glucan synthase (mU/50 μl)	
	Native	Trypsinized
+ EGTA[a]	0 00	0 42
+ EGTA + $MgCl_2$[b]	0 05	1 34
+ Ca^{2+}[c]	1 14	1 61
+ Ca^{2+} + $MgCl_2$	1.63	2.18
+ EGTA + poly-L-Orn[d]	1 26	2 35
+ Ca^{2+} + poly-L-Orn	2 81	2.82
+ EGTA + RR[e]	1 58	2 81
+ EGTA + RR + poly-L-Orn	1 87	3 25
+ Ca^{2+} + RR	2 26	2 77

[a] 1 6 mM
[b] 4 mM
[c] at the saturating concentration of 70 μM
[d] 25 μg/125 μl assay
[e] ruthenium red = RR, 0.36 mM

No further increase was observed when $MgCl_2$ was used in addition to poly-L-Orn or ruthenium red, present either alone or in combination with Ca^{2+} 1 6 mM EGTA allowed one to establish a $[Ca^{2+}] < 10^{-8}$ M, even in the presence of 4 mM $MgCl_2$

Table 2

Activation of the 1,3-β-D-glucan synthase by various polyamines and polycations

Substance added	1,3-β-D-Glucan synthase (mU/50 μl)	
	+ 2 5 μg[b]	+ 25 μg
Putrescine 2HCl	0 01	0 03
Spermidine 3HCl	0.03	0 08
Spermine 4HCl	0 07	0 32
Poly-L-Arg (15–70)[a]	0 66	0 65
Poly-L-Lys (30–70)	0 65	0 75
Poly-L-Orn (30–70)	0.84	1 12
Histone[c]	0 02	0 30
Chitosan (autoclaved)[d]	0 06	0 14

[a] Molecular mass range in kDa, poly-L-Arg contained 3–5% poly-L-Orn
[b] μg/125 μl assay; 2 5 μg spermine = 57 μM
[c] type II-S from calf thymus
[d] [2]

The enzyme was assayed under standard conditions with 1 6 mM EGTA to remove endogenous Ca^{2+} No significant influence was found by L-Lys, L-Arg, L-Orn and cytochrome *c*

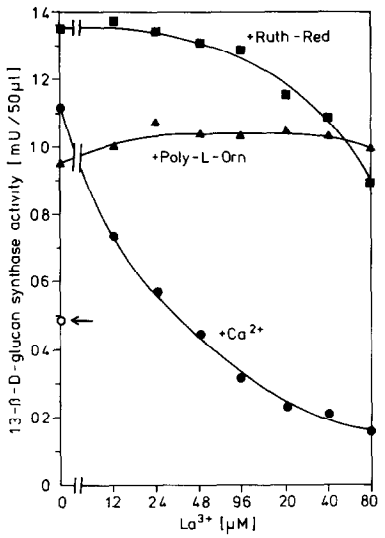

Fig.1 Influence of La^{3+} on 1,3-β-D-glucan synthase, activated by Ca^{2+} (70 μM, ●), poly-L-Orn (25 μg, ▲) or ruthenium red (0 36 mM, ■). Endogenous Ca^{2+} present under the latter 2 conditions was not zero but increased during the 10 min incubation time from below to slightly above (= about 2 μM) the detection limit of the Ca^{2+}-Selectrode Enzyme activity due to this endogenous Ca^{2+} is indicated by the arrow (○)

UDP-glucose [1], but allows rather low activity below 0.1 mM substrate (fig.3A) This could be slightly improved with 1 mM $MgCl_2$ Spermine, added alone at 57 μM, gives comparably small stimulation. Together with 5 μM Ca^{2+} its effect is, however, far more than additive, indicating a cooperative mode of action. Poly-L-Orn, present alone, is more active than spermine at the same weight but the cooperative effect with Ca^{2+} is less pronounced (fig.3B).

The polymer formed from UDP-glucose was rendered soluble on treatment [1] with an exo-1,3-β-D-glucanase from *Corticium rolfsii* by 80–90% and with an endo-1,3-β-D-glucanase from *Rhizopus arrhizus* by about 50–60% regardless of whether activation was effected by Ca^{2+}, poly-L-Orn or ruthenium red. This indicates that the major portion of the polymer formed from UDP-glucose consists of 1,3-β-D-glucan, similar to microsomes from other plants [4,5]. This conclusion was confirmed by direct determination of the 1,3-β-D-glucan using a newly developed quantitative fluorometric assay which is based on the

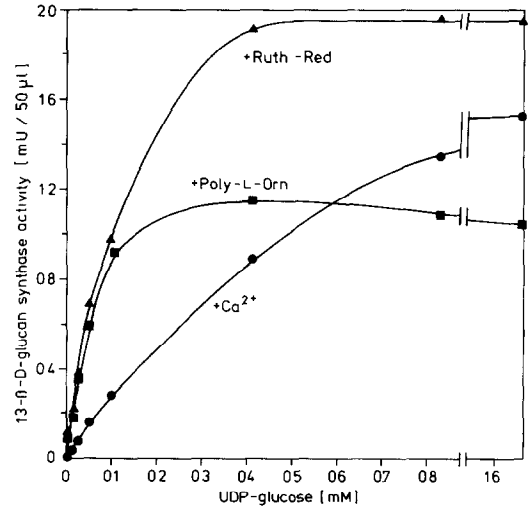

Fig 2 Dependence on substrate concentration of the 1,3-β-D-glucan synthase, activated by Ca^{2+} (70 μM, ●), poly-L-Orn (25 μg, ■) or ruthenium red (0 36 mM, ▲) For the latter 2 conditions 1 6 mM EGTA was also present to establish a $[Ca^{2+}] < 10^{-8}$ M

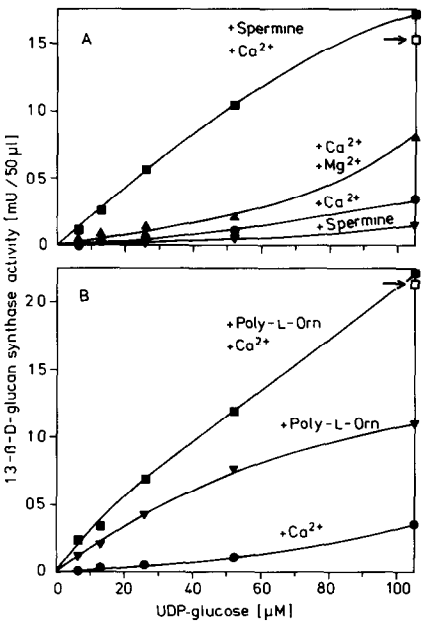

Fig.3 Cooperation of Ca^{2+} (5 μM) with other activators of 1,3-β-D-glucan synthase activity at various substrate concentrations. (A) Ca^{2+} alone (●), Ca^{2+} + 1 mM $MgCl_2$ (▲), spermine (2 5 μg/125 μl assay = 57 μM, + 1 6 mM EGTA, ▼, + Ca^{2+}, ■) Arrow Ca^{2+} + 1 mM $MgCl_2$ + 57 μM spermine (□) (B) Ca^{2+} alone (●), poly-L-Orn (2 5 μg, + 1 6 mM EGTA, ▼), Ca^{2+} + poly-L-Orn (■) Arrow· Ca^{2+} + 1 mM $MgCl_2$ + poly-L-Orn (□) The 5 μM Ca^{2+} were present endogenously Activity under standard assay conditions (70 μM Ca^{2+}, 0 8 mM UDP-glucose) was 1 8 mU/50 μl enzyme

aniline blue callose staining technique [2] (not shown) As the 1,3-β-D-glucanase preparations used were not absolutely free from other contaminating glucanases, no conclusions are possible on the presence or absence of minor portions of other linkages. This question can only be answered once results from methylation studies are available.

4. DISCUSSION

The 1,3-β-D-glucan synthase can be alternatively activated by Ca^{2+}, polyamines, polycationic proteins including histone, and by ruthenium red (tables 1 and 2). These substances exhibit very different chemical features but have in common the presence in the same molecule of many negatively charged groups. It appears likely that they all act principally by binding to positively charged sites, although in detail quantitative differences are evident. Ruthenium red, $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6$, is generally used as a cytochemical stain for polyanionic molecules (e.g. pectin). It is also commonly employed as an inhibitor of Ca^{2+} transport in mitochondria, where it appears to interact with 2 types of targets, one presumably being identical with the polar heads of phospholipids [6]. Poly-L-Lys can also interact with membrane phospholipids as indicated, for instance, by its ability to fuse liposomes or to cause membrane perturbation and lysis in many types of cells (see [2]).

Inhibitor studies have provided indirect evidence that the effect of Ca^{2+} on 1,3-β-D-glucan synthase is not mediated by calmodulin and that the enzyme requires phospholipids for its activity [1] This latter suggestion is sustained by our recent findings that various amphipathic substances at low concentrations can activate and at higher concentrations fully inhibit the enzyme, presumably by replacing endogenous phospholipids (submitted). It appears possible that alterations in the distribution of such lipids due to binding of the various activators used here may be the cause of their activating effect on 1,3-β-D-glucan synthase. Alternatively, direct interaction with negatively charged binding sites at the enzyme may change its conformation and improve its catalytic properties.

Ca^{2+} and the other activators appear to interact at different sites of the 1,3-β-D-glucan synthase as La^{3+}, which is known to be specific for many Ca^{2+} binding sites [7], can very effectively inhibit the activation by Ca^{2+}, but that effected by the others only scarcely (fig.1) The same conclusion is suggested by the observation that on trypsinization the Ca^{2+}-dependence of the enzyme nearly disappears whereas the effectiveness of poly-L-Orn and ruthenium red still increases (table 1).

Researchers interested in the fractionation of plant membranes often use the so-called glucan synthase II as a plasma membrane marker. This enzyme has usually been determined with 0.5 to 1 mM UDP-glucose due to its apparent low affinity (see [8]) and appears to be a Ca^{2+}-activated form of the enzyme, Ca^{2+} being present endogenously in the assay mixture (see fig.3). In contrast, the so-called glucan synthase I, a marker for Golgi membranes, is assayed at about 10 μM UDP-glucose

and thus appears to have a higher substrate affinity. However, this enzyme usually has extremely low activity compared to glucan synthase II, recognized when the values given in the literature (e g. [8]) as cpm [^{14}C]glucose incorporated are recalculated to give mU. This makes it unlikely that the very active high-affinity enzyme determined here in the presence of polycations or ruthenium red (fig.2) is the Golgi-located glucan synthase I Preliminary experiments also indicate that membranes bearing the 1,3-β-D-glucan synthase activated by poly-L-Orn and that activated by Ca^{2+} fully coincide on a sucrose density gradient (not shown) In addition, Ca^{2+} and especially spermine clearly cooperate as activators (fig.3) These 3 arguments sustain the assumption that Ca^{2+} and the other activators may exhibit their effects on the same enzyme.

The low activity at micromolar concentrations of UDP-glucose of the 1,3-β-D-glucan synthase II has been thought to indicate substrate activation (see [4,8]). The data in fig.3A show, however, that the affinity of the enzyme under these conditions can be greatly improved by spermine in combination with Ca^{2+} in the low micromolar range. Such a [Ca^{2+}] may occur in cells under stress situations which are known to lead to callose formation [2]. It appears possible, therefore, that spermine and the other polyamines could play an important role in callose synthesis. These substances are known to occur in plant cells in considerable amounts and their pool size is under hormonal control, a fact which has even led to the speculation that they may act as a type of 'second messenger' in plant cells [9]. Numerous physiological functions can be altered by exogenously applied polyamines; knowledge on their target enzymes is less profound [9]. Recently, the candidates have been propagated by soluble and membrane-located protein kinase [10]. We add 1,3-β-D-glucan synthase as a new example. The other activators used besides the polyamines clearly are artificial tools The effect of histone suggests, however, that an open mind should be kept with respect to endogenous regulative proteins bearing the appropriate clusters of basic amino acids.

ACKNOWLEDGEMENTS

This investigation was supported by grants from the Deutsche Forschungsgemeinschaft.

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