REGULATION OF PROLINE-INHIBITABLE GLUTAMATE KINASE (EC 2.7.2.11, ATP: γ-L-GLUTAMATE PHOSPHOTRANSFERASE) OF WINTER WHEAT LEAVES BY MONOVALENT CATIONS AND L-PROLINE

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Proline-inhibitable glutamate kinase (GK1) of winter wheat leaves is specifically regulated through feedback by L-proline during changes in the concentration of K^+- and Na^+-ions and in their ratio (K^+/Na^+). The enzyme is most active at 30°C at a relatively high $K^+ + Na^+$ concentration and a K^+/Na^+ ratio of 1·8 to 10·2 and at 0°C at both lower $K^+ + Na^+$ concentrations and a K^+/Na^+ ratio. An increase of the $K^+ + Na^+$ concentration to 1·25 mol/l and of the K^+/Na^+ ratio to 30·2 results in an activity decrease to one half; if, however, Na^+ is increased to make $K^+/Na^+ < 1$, the activity becomes one tenth of that of the control. GK1 is completely inhibited through feedback by 50 mmol/l L-proline in the medium at pH 7·2 containing 207 + + 40 mmol/l $K^+ + Na^+$; the inhibition is switched off by increasing the $K^+ + Na^+$ concentration to the optimum, *i.e.* 357 + 40 mmol/l and this is the condition necessary for the start of L-proline biosynthesis. An additional increase of the $K^+ + Na^+$ concentration in the presence of L-proline results in complete inhibition of the enzyme. The changes in the activity of GK1 comply with the recorded data on the changes of L-proline accumulation in plats which parallel the changes of K^+ and Na^+ concentration in the nutrient medium. The theory of GK1 regulation by L-proline during changes in the ionic strength of the medium, which had been designed n the preceding work, was experimentally confirmed in this study.

L-Proline, an imino acid, plays an important yet so far only little understood role in plants in the regulation of the basic elements of their metabolism, during ecological changes, and during ontogenesis[1]. It accumulates in plant tissues during dehydration at low temperatures[2,3], at increased ionic strength of the medium[4-8], drought[9], during photoperiods[10], and before the plants start to flower[11]. The decisive role in the intensity of L-proline biosynthesis under these conditions obviously plays the regulation of the first enzyme of L-proline biosynthesis from L-glutamate, *i.e.* of proline-inhibitable glutamate kinase *via* a feedback mechanism governed by L-proline[12]: the enzyme from winter wheat leaves was inhibited by 500—5 μmol/l concentrations of L-proline under optimal temperatures, pH-values, and ionic strengths of the medium[12]. When the temperature was decreased to 0°C inhibition immediately was switched over into allosteric activation; the latter is the decisive condition of the start and relative enhancement of L-proline biosynthesis. The dehydration of plants is a result of the decrease in free water content increasing the concentration of water soluble compounds[10]. The assumed mechanism of proline--inhibitable glutamate kinase regulation by L-proline[12] and of free proline accumulation in plant organs at increased concentrations of salts in the nutrient medium under optimal conditions[4-8]

have led to the deduction of a theory[12] that proline-inhibitable glutamate kinase switches off the inhibition through feedback by L-proline at optimal temperatures even at medium concentrations of monovalent ions and that the enzyme is inactivated by L-proline at high concentrations of these ions.

The soluble enzymes of halophytes and glycophytes are considerably sensitive to high electrolyte concentrations[13] yet the activity of the oligomer enzymes depends on the strength of the bond between the monomer[14]. The ionic strength of the enzyme media in the cell compartments considerably varies[15]: inside the cells are mostly K^+-ions, outside Na^+-ions. The plant enzymes are activated by K^+-ions and inhibited by Na^+-ions and their sensitivity to changes in the concentration of these ions and to their mutual interchange also varies; the experimental data, however, are meagre in this respect. Thus, *e.g.* proline-inhibitable glutamate kinase of winter wheat leaves is more active in potassium phosphate buffer[16] than in sodium phosphate buffer, glutamate dehydrogenase, glutamine synthase, and nitrate reductase of halophytes are strongly inhibited by increased Na^+ concentrations yet not by L-proline[17]. The effect of the K^+/Na^+ ratio on the activity of these enzymes has been little investigated so far. Na^+-ions can replace K^+-ions in plant cells to a low degree only (40—50 mmol/l, ref.[18]). So far we are lacking information on the concentration of K^+-ions and on the K^+/Na^+ ratio in cell compartments. The cytoplasm can become, however, enriched by Na^+- and Cl^--ions at higher NaCl concentrations of the nutrient medium[19]; the K^+/Na^+ ratios therefore considerably vary with the composition of the nutrient medium, organ, and its age[20], yet obviously they must be above 1 for the enzyme apparatus to perform its normal function. The adaptation of plant varieties to the increased ionic strength of the medium depends on the adaptability of the enzymes and obviously is connected with the properties of the variety.

In view of the important role played by L-proline in the system of metabolism regulation during the ontogeny of plants we have aimed to explain the general regularities of identical changes in biosynthesis intensity and in the accumulation of L-proline under different ecological conditions and during ontogenesis, of changes whose common cause is the decrease of the content of free water and thus the increase of the ionic strength of solutions in cells[10]. This paper reports on the results of the experiments investigating the effect of increasing concentrations and of the ratio of concentrations of monovalent ions, K^+ and Na^+, and of their combinations with L-proline on allosteric regulation of proline-inhibitable glutamate kinase in leaves of winter wheat (variety production. frost-resistant, drought-resistant Mironovska 808) under optimal and low temperature.

EXPERIMENTAL

Proline-inhibitable glutamate kinase was isolated and partly purified from acetone-dried leaves of 14-day plants of winter wheat, var. Mironovská 808, and assayed by the hydroxamate method[21] at optimal pH 7·2 using the procedure described in the preceding communication[16]. The composition of the basic incubation mixture is given in Table I. The incubation period was 90 ± 0·1 min at 30°C ± 0·1°C and 18 ± 0·01 h at 0°C ± 0·1°C. The activity of glutamate kinase is expressed as the change in absorbance at 540 nm calculated per 1 ml of enzyme solution under the conditions of the experiment. The results were evaluated statistically[22]. The concentration of K^+-ions was increased by the addition of KCl or K_2SO_4, the concentration of Na^+ by adding NaCl to the basic incubation mixture (Table I). The variants of experiments with final concentra-

tions of K^+- and Na^+-ions increased by the addition of KCl and NaCl are given in Tables II—IV, of experiments where K_2SO_4 was used to achieve the final concentrations of 250, 200, 150, and 50 mmol/l are shown in Fig. 1. The concentrations of the ions represent the sum of their concentrations in all components of the incubation mixture (Tables II—IV). In experiments designed to determine the effect of heavy metal ions Ag^+-ions were added to the basic incubation mixture as $AgNO_3$ to a final concentration of 250—2·5 mmol/l.

The chemicals used have been described in the preceding communications[12,16]; the remaining chemicals (KCl, NaCl, K_2SO_4 of analytical purity) were from Lachema (Czechoslovakia). $AgNO_3$ was purchased from Safina (Czechoslovakia).

RESULTS

Regulation of activity of proline-inhibitable glutamate kinase from winter wheat leaves as a result of change in concentration of K^+*-ions at* 30°C *and* 0°C: The activity of proline glutamate kinase was high in the basic incubation mixture (Table I) at 30°C containing 207·4 mmol/l K^+, 40 mmol/l Na^+, and 100 mmol/l Cl^-, the K^+/Na^+ ratio being 5·17. The activity increased to 126·7% of the control after the concentration of K^+-ions had increased to 407 mmol/l, yet it decreased to 69·2% after K^+-ions increased to 707 mmol/l and finally became only 55·5% of the control after the concentration of K^+-ions had reached 1 207 mmol/l (Table II). Similar results were obtained when the incubation was carried out at 0°C: the activity was high

TABLE I

Composition of basic incubation mixture for assays of proline glutamate kinase

Component	Volume ml	µmol in 1 ml of incubation mixture	pH	Total concentration mmol/l		
				K^+	Na^+	Cl^-
1. L-Glu	0·050	20	7·2[a]	30	—	—
2. ATP.2 Na^+	0·100	20	comp.	—	40	—
3. $NH_2OH.HCl$	0·050	100	7·2[a]	100	—	100
4. $MgSO_4.7\ H_2O$	0·050	20	comp.	—	—	—
5. K^+-phosphate buffer	0·100	25	7·2	43	—	—
6. water[b]	0·250	—	—	—	—	—
7. enzyme preparation	0·400	20 (buffer)	7·2	34·4	—	—
Total	1·000	45 (buffer)	7·2	207·4	40	100

[a] The solutions were neutralized to pH 7·2 by 8 mol/l KOH before the experiment; [b] in these experiments equal volumes of aqueous solutions of neutral salts and L-proline were pipetted (*cf.* Tables II—IV and Fig. 1).

TABLE II

Regulation of proline-inhibitable glutamate kinase from winter wheat (var. Mironovská 808) leaves by change in concentration of K^+-ions and of K^+/Na^+ ratio. The assay procedure is described under Experimental. *A* activity of enzyme expressed as change in absorbance at 540 nm calculated per 1 ml of enzyme preparation under the conditions of the experiment

Variant No	Temperature °C	mmol/l in incubation mixture				Ratio K^+/Na^+	$\bar{A}$	$\sigma_{\bar{x}}$ ($\times 10^4$)	$\sigma_{\bar{x}}^2$	% *A* to variant No 1
		KCl added	K^+ total	Na^+ total	Cl^+ total					
1	30	0	207·4	40	100	5·17	0·255	36·4	0·08	100·0
2		100	307·4	40	200	7·67	0·313	50·3	0·169	122·9
3		200	407·4	40	300	10·17	0·323	50·3	0·169	126·7
4		500	707·4	40	600	17·67	0·177	49·3	0·162	69·2
5		1 000	1 207·4	40	1 100	30·17	0·142	41·6	0·115	55·7
1	0	0	207·4	40	100	5·17	0·145	30	0·143	100·0
2		50	257·4	40	150	6·43	0·188	50	0·166	129·3
3		100	307·4	40	200	7·67	0·145	0·0	0·0	100·0
4		200	407·4	40	300	10·17	0·080	40	0·107	55·2
5		500	707·4	40	600	17·67	0·075	20	0·027	51·7
6		1 000	1 207·4	40	1 100	30·17	0·060	20	0·027	41·4

TABLE III

Regulation of proline-inhibitable glutamate kinase from winter wheat (var. Mironovská 808) leaves by change of concentration of Na^+-ions and K^+/Na^+ ratio. The assay procedure is described under Experimental. *A* activity of enzyme expressed as change in absorbance at 540 nm calculated per 1 ml of enzyme preparation

Variant No	Temperature °C	mmol/l in incubation mixture				Ratio K^+/Na^+	$\bar{A}$	$\sigma_{\bar{x}}$ ($\times 10^4$)	$\sigma^2_{\bar{x}}$	% *A* to variant No 1
		KCl added	K^+ total	Na^+ total	Cl^- total					
1	30	0	40	207·4	100	5·17	0·255	34·6	0·08	100·0
2		75	115	207·4	175	1·80	0·399	70·2	0·329	156·4
3		225	265	207·4	325	0·78	0·128	26·5	0·467	50·0
4		750	790	207·4	850	0·26	0·032	23·1	0·036	12·4
1	0	0	40	207·4	100	5·17	0·145	141·4	1·00	100·0
2		75	115	207·4	175	1·80	0·275	141·4	1·00	189·7
4		225	265	207·4	325	0·78	0·088	0·0	0·0	60·3
4		750	790	207·4	850	0·26	0·15	282·8	4·00	10·3

TABLE IV

Regulation of proline-inhibitable glutamate kinase of winter wheat (var. Mironovská 808) leaves by 50 mmol/l L-proline during increase in concentration of K^+- and Na^+-ions and K^+/Na^+ ratio at 30°C. The assay procedure is described under Experimental. *A* activity of enzyme expressed as change in absorbance at 540 nm and calculated per 1 ml of enzyme preparation under the conditions of the assay.

Variant No	mmol/l in incubation mixture				Ratio K^+/Na^+	*A*, in variant			
	KCl added	K^+ total	Na^+ total	Cl^- total		without L-Pro	% of var. No 1	with L-Pro	% of var. without L-Pro
1	0	207·4	40	100	5·17	0·400	100·0	0·000	0·00
2	50	257·4	40	150	6·43	0·325	81·3	0·000	0·00
3	100	307·4	40	200	7·67	0·450	112·5	0·250	55·6
4	150	357·4	40	250	8·93	0·400	100·0	0·400	100·0
5	200	407·4	40	300	10·17	0·250	62·5	0·000	0·00
6	500	707·4	40	600	17·67	0·150	37·5	0·000	0·00
	NaCl added								
7	0	207·4	40	100	5·17	0·400	100·0	0·000	0·00
8	70	207·4	110	170	1·80	0·150	37·5	0·450	300·0

in the control experiments where the concentration of K^+- and Na^+-ions was 207 and 40 mmol/l, resp., the K^+/Na^+ ratio being 5·17. An increase in K^+ to 257 mmol/l and of the K^+/Na^+ ratio to 6·4 resulted in maximal activity, *i.e.* 129·3% of the control, however, after K^+ had been raised to 307 mmol/l and the K^+/Na^+ ratio to 7·67 the activity was the same as that of the control and became 41·4% of the control after K^+ had been raised to 1 207 mmol/l and the K^+/Na^+ ratio to 30·2 (Table II).

Regulation of activity of proline-inhibitable glutamate kinase of winter wheat leaves by change of concentration of Na^+*-ions at* 30°C *and* 0°C: The control sample again contained 207 mmol/l K^+ and 40 mmol/l Na^+, their ratio being 5·17 (Table III). The activity of the enzyme increased to 156·3% at 30°C after the Na^+-concentration had been raised to 115 mmol/l and the K^+/Na^+ ratio thus decreased to 1·8. An additional increase in Na^+ to 265 mmol/l and decrease of the K^+/Na^+ ratio of 0·78 made the activity decrease to 50%; after Na^+ had become 790 mmol/l and the K^+/Na^+ ratio 0·26 the activity dropped to 12·4% of the control (Table III). The enzyme activity varied during incubation at 0°C like at 30°C yet the increase to a maximum at total concentration of Na^+-ions 115 mmol/l and a K^+/Na^+ ratio equal 1·8 was more marked (189·7%); the same holds for the decrease to a minimum (10·3% of control, Table III) at the highest Na^+ concentration used.

Regulation of proline-inhibitable glutamate kinase from winter wheat leaves by increasing the ionic strength of the medium by potassium sulfate: The additions of K_2SO_4 to the basic incubation mixture (Table I) in concentrations of 250 – 2·5 mmol/l (Fig.1), *i.e.* additions increasing total K^+ and Na^+ from 207 and 40 mmol/l resp. to 707 and 40 mmol/l resp., gradually and irregularly decreased the activity of proline-inhibitable glutamate kinase (Fig. 1).

Regulation of proline-inhibitable glutamate kinase from winter wheat leaves by Ag^+*-ions*. Proline-inhibitable glutamate kinase was completely inhibited by the

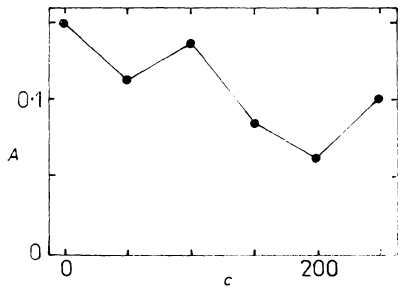

FIG. 1

Regulation of proline-inhibitable glutamate kinase of winter wheat (var. Mironovská 808) leaves controlled by increasing concentration of K^+-ions and affected by the addition of potassium sulfate to the basic incubation mixture (Table I). The assay procedure is described under Experimental. *A* activity of enzyme expressed as change in absorbance at 540 nm calculated per 1 ml of enzyme preparation under the conditions of the assay. *c* final concentration of K_2SO_4 in mmol/l in the basic incubation mixture

addition of Ag^+-ions in concentration of 250–2·5 mmol/l to the basic incubation mixture (Table I, the activity of the control was 0·150).

Regulation of proline-inhibitable glutamate kinase from winter wheat leaves by monovalent ions and L-*proline.* The effect of increasing the concentration of K^+- and Na^+-ions and of changes in the K^+/Na^+ ratio on the regulation of proline-inhibitable glutamate kinase through feedback by L-proline at optimal temperature of 30°C was examined. The activity changes in the experiment where the concentration of K^+-ions and the K^+/Na^+ ratio increased were similar in controls without proline (Table IV) to the activity changes observed in the series of preceding experiments (Table II and III). L-Proline when present in 50 mmol/l concentration in the incubation mixtures completely inhibited proline-inhibitable glutamate kinase through the feedback mechanism when of $K^+ = 207$ and $Na^+ = 40$ mmol/l (K^+/Na^+ ratio 5·7) and $K^+ = 257$ and $Na^+ = 40$ mmol/l (K^+/Na^+ 6·43). An additional increase of K^+ to 307 mmol/l and of K^+/Na^+ to 7·67 switched off partly the inhibition whereas a complete switch off was achieved at 357 mmol/l K^+ and K^+/Na^+ equal 8·93. An increase of the concentration of K^+ to 407 mmol/l and up and of K^+/Na^+ to 10·17 and up resulted in complete inhibition of the enzyme in the presence of L-proline (Table IV). The concentration of Na^+-ions increased to 110 mmol/l and the thus decreased K^+/Na^+ ratio of 1·89 switched off inhibition through feedback by L-proline and moreover activated the enzyme to triple the activity of the control in the absence of L-proline (Table IV).

DISCUSSION

According to recorded data L-proline plays the decisive role in the regulation of important elements of plant metabolism[1], especially in the process of flowering[11], in the photoperiod[10], in biochemical adaptation of plants to low temperatures[2,3,23–25], to drought[9], and to increased ionic strength of the medium[4–8]. In the course of these processes bound proline[3] and later also free proline[1–11] accumulates as a result of dehydration and thus of increased ionic strength of cytoplasm solutions. This accumulation is a result of the enhancement of L-proline biosynthesis from L-glutamate[3] whose intensity is obviously governed by allosteric regulation of the first enzyme, *i.e.* the proline-inhibitable glutamate kinase, by the end product, L-proline[12]. The structure of the enzyme is well suited for this purpose: it is an oligomer with relatively firmly associated monomers[12] which is thus very active at various temperatures up to high ionic strengths (Table II and III, Fig. 1) and equally at low temperatures (–20°C, ref.[12]). The activity of the enzyme decreases at high ionic strength of the medium and in the absence of L-proline on the average to one half of the activity of the control (Table II and III). The optimal value of ionic strength decreases as a result of the relative solubility of salts of monovalent ions[30] with the decreasing temperature: it is lower at 0°C than at 30°C (Table II and III).

The activity of proline-inhibitable glutamate kinase depends not only on the concentration of $K^+ + Na^+$ but also on the K^+/Na^+ ratio. The results of our experiments permit us to conclude that the activity of the enzyme is maintained at a value close the optimal value over a wide range of K^+/Na^+ ratios, from 1·8 to 10·17, yet is the highest at 1·8 (Table II and III), in accordance with the recorded data on the situation *in vivo*[18,19]. Na^+-ions are more active than K^+-ions, obviously because of their higher chemical activity, even in the range of high concentrations where they cause a more marked decrease of the reaction rate of proline-inhibitable glutamate kinase at low concentrations, especially if the K^+/Na^+ ratio is deep below 1 (Table III). This shows that proline-inhibitable glutamate kinase of wheat leaves needs for the reaction rate to proceed at optimal rate a certain optimal concentration of $K^+ + Na^+$ and a certain K^+/Na^+ ratio; only then the enzyme can maintain its optimal conformation and the stability of the oligomer. The activity of the oligomer is the highest at optimal $K^+ + Na^+$ concentration, the K^+/Na^+ ratio being $1-2$, and decreases more markedly when the K^+/Na^+ ratio drops below 1 and Na^+ increases than when $K^+/Na^+ > 10$ and the concentration of K^+ increases (Tables II and III). This points to the known importance of K^+-ions in plant nutrition[31] yet also to the importance of Na^+-ions for the optimal rate of the enzymatic reaction, a finding which has also been quoted in literature[18,19].

The type of anion obviously affects the rate of the reaction catalyzed by proline-inhibitable glutamate kinase less than the type of cation; this follows from the replacement of Cl^--ions by SO_4^{2-}-ions (Fig. 1, Tables II and III). The complete inhibition by Ag^+, a heavy metal ion, indicates that the enzyme has catalytic SH-groups in the substrate active center which are blocked by Ag^+-ions[24].

The course of the regulation of the rate of reactions catalyzed by proline-inhibitable glutamate kinase from winter wheat leaves considerably varies with changes in the concentration of K^+- and Na^+-ions at optimal temperature if L-proline is present. We have shown in the preceding study[12] that proline-inhibitable glutamate kinase in solutions at optimal pH (7·2, ref.[16]) containing a considerable concentration of K^+- and Na^+-ions, identical with the concentrations used in controls in this study (Tables II and III), is inhibited through feedback by L-proline. According to the theory outlined before[12], the hydrophobic part of the ring of L-proline, an allosteric effector, is bound to the effector site of the enzyme by hydrophobic forces whereas the ionic part of the molecule by ionic forces. If the temperature drops to 0°C, the concentration of K^+ and Na^+-ions remaining unchanged, the hydrophobic link[14] between L-proline and the effector site loosens, the conformation of the enzyme changes as a result of cooperations and allosteric inhibition is switched over into activation. The results of this study show that proline-inhibitable glutamate kinase of winter wheat leaves changes the type of regulation through feedback by L-proline also under the optimal temperature of 30°C with the changing ionic strength of the incubation medium: it switches over the inhibition by L-proline to activation after

a certain, optimal concentration of $K^+ + Na^+$ has been achieved and is completely inactivated by additional increase of the concentration of $K^+ + Na^+$ in the presence of L-proline (Table IV). All these experiments permit us to conclude that the character of regulation of proline-inhibitable glutamate kinase through feedback by L-proline and thus the intensity of L-proline biosynthesis in plants at various temperatures are virtually governed by the level of the concentration of $K^+ + Na^+$ at a certain K^+/Na^+ ratio. Lower temperatures by merely decreasing the relative solubility of K^+- and Na^+-salts and by loosening the hydrophobic link of L-proline with the effector site[12] facilitate the switch over of the inhibition through feedback by L-proline to activation and decrease the optimal concentration of $K^+ + Na^+$ necessary for this switch over (Table IV, ref.[12]). This immediate, instantaneous transition of the type of regulation of proline-inhibitable glutamate kinase, brought about by cooperative changes in the oligomer conformation, can be *in vivo* the result of an increase of the concentration of monovalent ion salts (Table IV) proceeding either directly by the uptake of K^+ and Na^+ from a nutrient medium of higher ionic strength[4-8,26-29] or due to losses of free water during dehydration of plant tissues[10] in dryness[9], cold[2,3,25], during the photoperiod[10], before the flowering[11], *etc.* The total inhibition of proline-inhibitable glutamate kinase of winter wheat leaves by L-proline at a K^+ and Na^+ concentration higher than the optimal concentration of the inhibition to activation switch over (Table IV) corresponds to a complete displacement of L-proline from the ionic link with the effector site and to the deformation of the oligomer and its substrate sites[12]. The instantaneous switch over of the regulation type of proline-inhibitable glutamate kinase under optimal conditions *in vitro* (Table IV), which starts L-proline biosynthesis, is entirely consistent with the changes in the biosynthesis and accumulation of L-proline observed in numerous *in vivo* experiments[4-8,26-29]: free L-proline is intensively accumulated in organs of various plants at optimal temperatures only on condition that the concentration of KCl or NaCl in the nutrient medium was considerably high, not at higher or lower concentrations. Concentrations of K^+ and Na^+ exceeding those which are optimal for the activation of proline-inhibitable glutamate kinase by L-proline (Table IV) correspond *in vivo* to the salinity of the medium which stops L-proline biosynthesis[4-8,26-29] and brings damage to the plants[31].

The results of the experiments of this (Table IV) and the preceding studies[12,16] show that the changes in proline-inhibitable glutamate kinase of plants through feedback by L-proline during the changes in the concentration of monovalent ions, $K^+ + Na^+$ and of the K^+/Na^+ ratio play the decisive role in the regulation of L-proline biosynthesis in plants. All factors which lead to changes in the concentration of $K^+ + Na^+$ and in the K^+/Na^+ ratio affect these regulations.

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