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Pyrroline-5-Carboxylate Reductase in *Chlorella autotrophica* and *Chlorella saccharophila* in Relation to Osmoregulation[1]

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ABSTRACT

Pyrroline-5-carboxylate (P5C) reductase (EC 1.5.1.2), which catalyzes the reduction of P5C to proline, was partially purified from two *Chlorella* species; *Chlorella autotrophica*, a euryhaline marine alga that responds to increases in salinity by accumulating proline and ions, and *Chlorella saccharophila*, which does not accumulate proline for osmoregulation. From the elution profile of this enzyme from an anion exchange column in Tris-HCl buffer (pH 7.6), containing sorbitol and glycine betaine, it was shown that P5C reductase from *C. autotrophica* was a neutral protein whereas the enzyme from *C. saccharophila* was negatively charged. The kinetic mechanisms of the reductase was characteristic of a ping-pong mechanism with double competitive substrate inhibition. Both enzymes showed high specificity for NADH as cofactor. The affinities of the reductases for their substrates did not change when the cells were grown at different salinities. In both algae, the apparent K_m values of the reductase for P5C and NADH were 0.17 and 0.10 millimolar, respectively. A fourfold increase in maximal velocity of the reductase was observed when *C. autotrophica* was transferred from 50 to 150% artificial sea water. Even though the reductase was inhibited by NaCl, KCl, and proline, it still showed appreciable activity in the presence of these compounds at molar concentrations. A possible role for the regulation of proline synthesis at the step catalyzed by P5C reductase is discussed in relation to the specificity of P5C reductase for NADH and its responses to salt treatments.

Δ-Pyrroline-5-carboxylate reductase (L-Proline:NAD(P)-5-oxidoreductase, EC 1.5.1.2) catalyzes the final step in the biosynthetic pathway leading from glutamic acid to proline. The enzyme has been partially purified and characterized for many higher plants (7, 9, 13, 17, 21, 23). None of these studies suggest a major role for P5C[2] reductase in the regulation of proline biosynthesis. However, the activity of P5C reductase has been shown to increase 3.5-fold when seedlings of *Pennisetum typhoides* were grown in the presence of 17 mM NaCl with a concomitant increase in proline concentration (7). More recently, Treichel (23) showed that P5C reductase activity and substrate affinity increased with progressive adaptation to NaCl stress of cell suspension cultures of *Mesembryanthemum nodiflorum*. Cells grown in 400 mM NaCl showed nearly 4 times more activity of P5C reductase than cells cultivated in the absence of NaCl. Moreover, pyrroline-5-carboxylate occupies a central role not only in the biosynthesis and oxidation of proline, but also in the metabolism of ornithine and arginine via the amination of glutamate semialdehyde by ornithine transaminase (22). Thus, any variation in the activity of P5C reductase will potentially affect several metabolic pathways.

In erythrocytes there is substantial evidence for a role for P5C reductase in the transfer of redox potential (15). In these cells, glucose-6-phosphate dehydrogenase, which catalyzes the rate-limiting step of the pentose phosphate pathway, is dependent on the availability of $NADP^+$ and is inhibited by NADPH. It is believed that P5C reductase by reducing P5C to proline with NADPH as cofactor serves to furnish $NADP^+$ to glucose-6-phosphate dehydrogenase for the synthesis of purine nucleotides. Recently, it has been shown that in soybean nodules P5C reductase can indeed play an important role for the support of purine biosynthesis in addition to producing proline for incorporation into protein (8). In both erythrocytes and soybean nodules the P5C reductases are characterized by a higher affinity for NADPH than NADH, and a sensitivity to inhibition by $NADP^+$ but not by proline. Finally, as pointed out by Bellinger and Larher (2), in glycophytes and in some halophytes subjected to water stress, proline accumulation could play a role of redox buffer by storage of excess reductants in a nontoxic form in the cytosol.

In light of these recent findings, we decided to investigate the kinetic properties of P5C reductase in two different species of *Chlorella*: *autotrophica* and *saccharophila*. *C. autotrophica* is a euryhaline marine alga that lives in estuaries, tide pools, or brackish water ponds. It osmoregulates by adjusting its concentrations of inorganic ions and proline, and can grow over a salinity range of 1 to 400% ASW (1). *C. saccharophila* is a terrestrial alga that has been isolated from aerial localities and the sap of trees. It does not accumulate high amounts of proline for osmoregulation and 175% ASW represents its upper limit of salinity tolerance. Due to their intrinsic differences in capacities for proline accumulation and salinity tolerance, these species were chosen for the determination of kinetic properties of their P5C reductases.

MATERIALS AND METHODS

Growth of Algae

Chlorella autotrophica Shihira and Krauss (clone 580 obtained from Dr. R. R. L. Guillard, Woods Hole Oceanographic Inst., Woods Hole, MA Culture Collection) and *Chlorella saccharophila* (UTEX clone 2469; originally CCAP 211/

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[2] Abbreviations: P5C, Δ-pyrroline-5-carboxylate; ASW, artificial sea water; FPLC, fast protein liquid chromatography.

9a) were grown axenically on an ASW medium prepared according to McLachlan (11) with 2 mM ammonium as nitrogen source. For example, a 50% ASW solution contains 200 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM $MgSO_4$, plus nutrients and vitamins, for a total salt concentration of 230 mM. Cells were grown at 18°C on a 12/12 h light/dark cycle (24 $W \cdot m^{-2}$ from Daylight fluorescent tubes). Cell counting was done with a hemacytometer and the experiments were carried out with cells from late exponential phase cultures.

Preparation and Determination of Pyrroline-5-Carboxylate

P5C was prepared according to the method of Williams and Frank (25) based on the periodate oxidation of D-L-δ-hydroxylysine. The reaction mixture was applied to a Dowex 50 column equilibrated with water after washing with 1 N HCl. Elution of P5C was started with 1 N HCl and the appropriate fractions were pooled, yielding a 24 mM solution of DL-P5C. At low pH and temperature, P5C was stable for several months. Prior to utilization for enzyme assay, P5C was neutralized with 1 N NaOH and kept on ice. Quantitative assays of P5C were performed in the presence of *o*-aminobenzaldehyde or with ninhydrin (25).

Preparation of Cell-Free Extracts

One L of culture was harvested by centrifugation at 7,000*g* for 5 min at 10°C. The cell pellet was washed twice in nitrogen-free ASW medium and resuspended in 3 to 4 mL of extraction buffer (10 mM $MgSO_4$, 0.5 mM EDTA, 1 mM DTT, 10 mM mercaptoethanol, 300 mM sorbitol, 1% glycine betaine, and 25 mM Tris-HCl [pH 7.6]). Cells were broken by two passages through a French Pressure Cell at 5°C and 200 MPa. After centrifugation at 25,000*g* for 30 min at 5°C, the supernatant was filtered through a 0.22 μm Millipore filter (Millex-GV). A 2 mL aliquot was applied on a Pharmacia FPLC column (Mono-Q, anion-exchange) and fractionated using a linear gradient of 0 to 400 mM NaCl. Fractions (1.0 mL) were collected every minute and assayed for P5C reductase activity as described below. The elution buffers contained 25 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT, 10 mM mercaptoethanol, 300 mM sorbitol, 1% glycine betaine, and 0 or 1 M NaCl.

P-5-C Reductase and Proline Dehydrogenase Assays

For routine assays of enzyme activity, the stock solution of P5C (about 12 mM L-P5C in 1 N HCl) was neutralized with 1 N NaOH and kept on ice. The reaction mixture contained 0.9 mM of neutralized L-P5C, 0.28 mM NADH, 150 mM NaCl, and 10 to 20 μL of enzyme in a total volume of 760 μL of 50 mM potassium phosphate buffer (pH 7.2). The oxidation of NADH was monitored at 340 nm at room temperature for 3 to 4 min. Without P5C in the reaction mixture, the oxidation of NAD(P)H was never more than 8.0% of the activity shown in the presence of P5C. The proline dehydrogenase activity was monitored with 20 mM-proline and 5 mM NAD^+ in NaOH-glycine buffer (pH 10.4). Protein was determined with the standard assay of Bio-Rad Protein Assay Dye Reagent using BSA as standard proteins.

RESULTS

Purification of Pyrroline-5-Carboxylate Reductase

Pyrroline-5-carboxylate reductases of *Chlorella autotrophica* and of *Chlorella saccharophila* were partially purified in one step by FPLC on a Pharmacia Mono Q anion exchange column. In the presence of thiol reagents and of the stabilizing agents, sorbitol and glycine betaine, a 40-fold and a 15-fold enrichment of the enzymes from *C. saccharophila* and *C. autotrophica*, respectively, were achieved with no loss of activity as compared to the crude extract. However, very different elution profiles of the reductase of *C. autotrophica* and *C. saccharophila* were obtained. P5C reductase from *C. autotrophica* did not bind to the anion exchange column and eluted in front of the NaCl gradient; mainly in fractions 2-3-4 (Fig. 1). In contrast, the P5C reductase from *C. saccharophila* grown under similar conditions, eluted in fractions 18-19-20 at 260 mM NaCl indicating that the protein was highly negatively charged at pH 7.6 (Fig. 1). The elution profile of the reductase of *C. autotrophica* in the presence of stabilizing agents indicated that the protein was either neutral or posi-

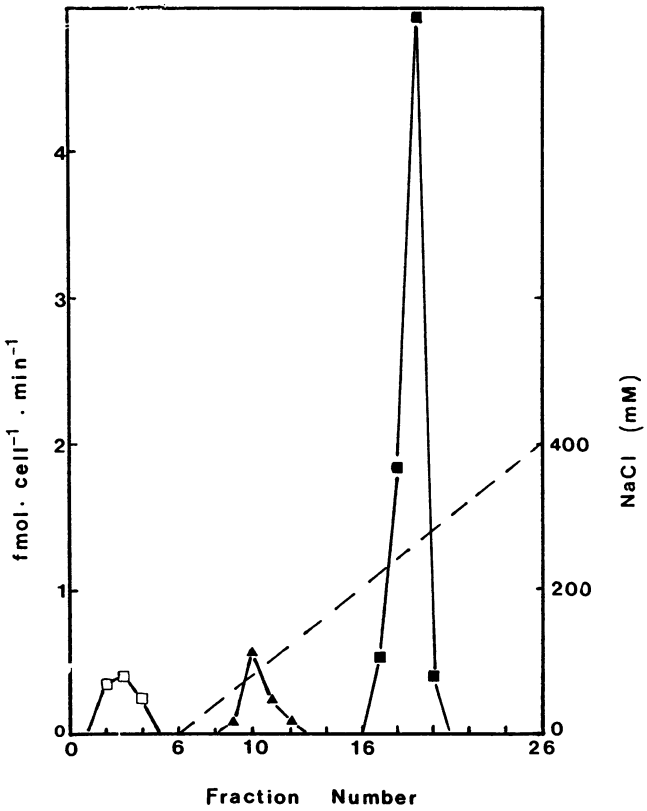
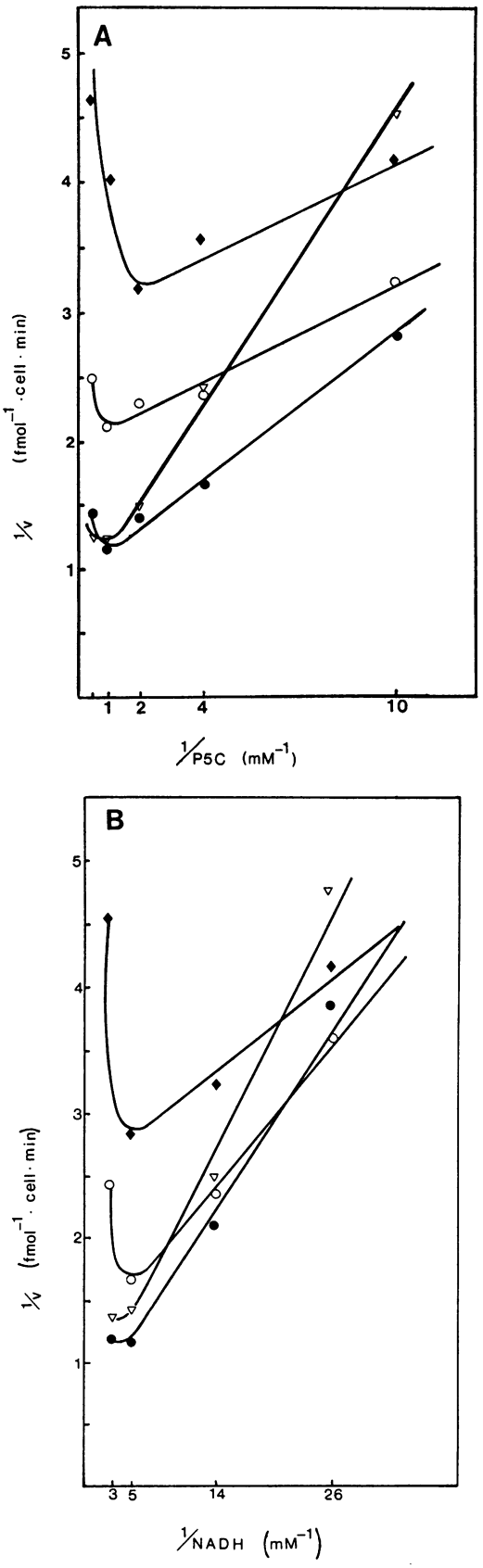


Figure 1. Elution profile of P5C reductase from ion exchange FPLC using a Pharmacia Mono Q column. The reductase of *C. autotrophica* was eluted in Tris-HCl buffer (pH 7.6) in the absence (▲) or the presence (□) of stabilizing agents sorbitol and glycine betaine. The enzyme from *C. saccharophila* was eluted in the presence of stabilizing agents (■). Enzyme activities are expressed in fmol/cell/min and the dotted line represents the NaCl gradient.



tively charged. In order to differentiate between these two alternatives, the fractions 2-3-4 eluted from the anion exchange column were combined and loaded on a cation exchange column (Mono-S) and eluted with a 400 mM NaCl gradient in 25 mM Hepes buffer (pH 7.6). The enzyme was found in the very first fractions of the gradient showing that the P5C reductase was neutral in that particular buffer (results not shown). Interestingly, when the P5C reductase of *C. autotrophica* was chromatographed in 25 mM Tris-HCl buffer pH 7.6 without the presence of sorbitol and glycine betaine, the enzyme acquired an overall negative charge and eluted in fractions 9-10-11 at 85 mM NaCl (Fig. 1). However, the yield was low and variables (from 50–85%). These results indicate that the quarternary structure of the enzyme is drastically changed by the buffer environment. In all subsequent experiments, Tris-HCl buffer with the addition of thiol reagents and stabilizing agents, sorbitol and glycine betaine were used to achieve optimum yields.

Enzyme Kinetics and pH Optimum

To characterize the kinetic mechanisms of the reductase, the initial rates of P5C reduction were calculated using five different NADH concentrations (from 40–300 μM) and five different L-P5C concentrations (from 25–2000 μM). Double reciprocal plots of velocity against P5C concentration (NADH concentration held constant) and of velocity against NADH concentration (P5C concentration held constant) generated a set of curves characteristic of a ping-pong mechanism (also called substituted-enzyme mechanism) with double competitive substrate inhibition. In this type of mechanism, both substrates form dead-end complexes with the wrong enzyme form (20). When either substrate is varied at different fixed levels of the other, the Lineweaver-Burk plots curve up as they approach the 1/v-axis and the minimum point of the curve moves closer to the 1/v-axis as the concentration of the fixed substrate increases. However, from the primary Lineweaver-Burk plots of data, it was obvious that the inhibition constant for P5C was above 1.0 mM and the one for NADH was over 300 μM (Fig. 2). In all probability, these substrate concentrations are unphysiological, so it was decided to use 900 μM P5C and 280 μM NADH for all subsequent experiments.

As shown in Figure 3, the P5C reductases from both *C. autotrophica* and *C. saccharophila* had a broad pH optima between 7.0 and 8.0. A pH of 7.2 was routinely used for all subsequent assays, because P5C is more stable at that pH than at pH 8.0. The low activity observed at pH 9.0 indicates that, *in vivo*, the enzyme functions as a reductase rather than a dehydrogenase. Even though the enzyme of *C. autotrophica* did show a low level of NAD^+-dependent activity (about 0.02 fmol/cell/min) at pH 10.4, this reaction is certainly unphysiological, and results from the displacement of the mass action ratio of the reductase reaction (the P5C reductase of *C. saccharophila* was not tested for dehydrogenase activity).

Figure 2. Lineweaver-Burk plot of P5C reductase activity of *C. autotrophica*. A, 1/v *versus* 1/P5C at fixed concentrations of NADH; 330 μM (▽), 200 μM (●), 71 μM (○), and 37 μM (◆). B, 1/v *versus* 1/NADH at fixed concentrations of P5C; 2000 μM (▽), 1000 μM (●), 250 μM (○), and 100 μM (◆).

Because the kinetic properties of P5C reductase have been shown to change in some plants subjected to salinity stress (7, 23), the maximal activity and the apparent K_m for P5C and NADH were investigated at low (50% ASW, corresponding to 230 mM salts) and high salinity (150% ASW, corresponding to 630 mM salts) in the two *Chlorella* species, which respond quite differently to salinity stress; *C. autotrophica* being much more salt tolerant than *C. saccharophila*. Surprisingly, their physiological difference toward salinity stress was not reflected in the affinity of their reductases toward P5C or NADH. In both algae, the apparent K_m values of P5C reductase for P5C and NADH were not significantly different at either salinity (Table I). K_m for P5C was about 0.17 mM and for NADH it was equal to about 0.10 mM. On the other hand, the maximal activity of the reductase increased 4-fold when *C. autotrophica* was grown at 150% ASW as compared to 50% ASW (Table I). Only a small increase of about 1.4-fold was observed for P5C reductase in *C. saccharophila* grown at higher salinity. At either salinity, the P5C reductase of *C. saccharophila* has a higher maximal activity, on a protein basis, than *C. autotrophica*.

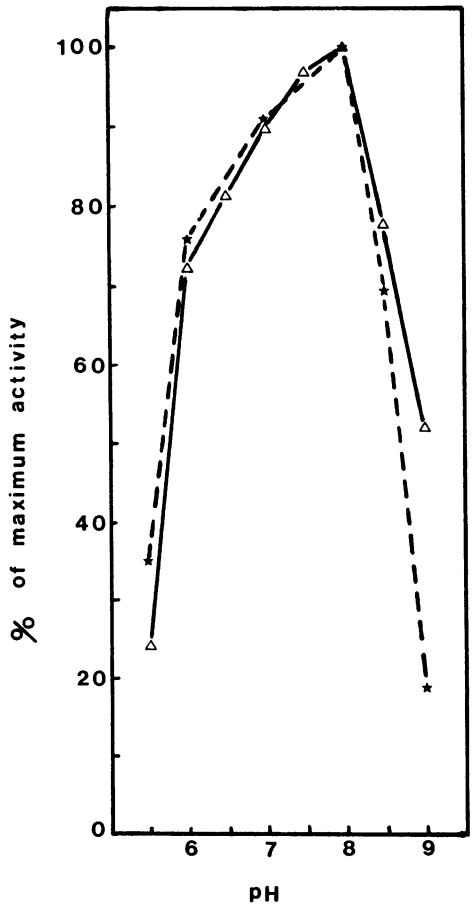


Figure 3. P5C reductase activity of *C. autotrophica* (△) and *C. saccharophila* (★) in function of pH. Enzyme activities were measured in 50 mM acetate buffer (pH 5.5, 6.0, and 6.5), 50 mM phosphate buffer (pH 7.0, 7.2, and 7.5) or 50 mM Tris-HCl buffer (pH 8.0, 8.5, or 9).

Effects of Salt Concentrations and Proline on Enzyme Activity

It has been shown that *C. autotrophica* cells grown at 300% ASW (1.26 M salts) contain about 1.6 M proline (1). The P5C reductase of this alga must thus be able to function in the presence of high levels of salts and proline. Table II shows that this was indeed the case. Even though the reductase was inhibited by NaCl and KCl, it still had about 50% of the control activity at 0.5 M NaCl, and 2 M NaCl only inhibited about 70% of the activity (Table II). In the presence of 0.5 M proline, the rate of P5C reduction was 40% of the control value, and this rate went down to 26 and 11% at 1.0 and 2.0 M proline, respectively. This inhibition was certainly caused by a displacement of the mass action ratio of the reaction at high proline concentration, rather than by a detrimental effect of proline itself on the structure of the enzyme. We also observed that the inhibition caused by NaCl was not linear over the range of P5C concentrations used. In general, for a given amount of NaCl, the inhibition of P5C reductase activity was highest at the lowest P5C concentration used. For example, at 330 mM NaCl, the activity of P5C reductase was 90% of the control in the presence of 1.25 mM P5C but only 72% in the presence of 0.3 mM P5C (data not shown).

Pyridine Nucleotide Specificity

One of the characteristics of P5C reductase isolated from *Chlorella*, compared to those from higher plants, was its specificity for NADH as a cosubstrate. In the presence of NADPH from 50 to 300 μM, at pH 7.2, the reductase did not yield an activity exceeding 10% that observed with NADH. At low NADPH concentrations the activity observed was negligible (data not shown). Because other reductases that are considered to be specific for NADH have much higher relative activity with NADPH at a lower pH value (6), we investigated the reduction of NADPH from pH 5.0 to 7.0. Again, the maximum activity observed was never more than 10% that with NADH (results not shown). This high specificity for NADH indicates that in *Chlorella*, P5C reductase is unlikely to play a role in the regulation of purine biosynthesis via the formation of $NADP^+$. This was further confirmed by examining the effects of NAD^+ or $NADP^+$ on the NADH-dependent reduction of P5C. Whereas increasing concentrations of $NADP^+$ had virtually no effect on the enzyme (Table III), the rate of reaction was strongly influenced by the NAD^+/NADH ratio. At a NAD^+/NADH ratio of 55, the rate of reduction of P5C was only 38% that of the control rate in the absence of NAD^+ (Table III).

DISCUSSION

P5C reductases from *Chlorella autotrophica* and *Chlorella saccharophila* in Tris-HCl buffer at pH 7.6 containing thiol reagents and stabilizing agents were quite different. While the P5C reductase from *C. autotrophica* was neutral under these conditions, the *C. saccharophila* enzyme was highly negatively charged. Furthermore, the P5C reductase from *C. autotrophica* acquired an overall negative charge in the absence of sorbitol and glycine betaine while its activity decreased by

Table I. *Effect of Salinity on Maximal Activity (V_{max}) and K_m of P5C Reductase of C. autotrophica and C. saccharophila*

K_ms for NADH were estimated from 10 different NADH concentrations (from 20–280 μM) at 900 μM P5C. K_ms for P5C were estimated from 10 different concentrations of P5C (from 25–900 μM) at 280 μM NADH. Kinetic parameters were determined by linear regression and the coefficient of determination (r^2) was never less than 0.998. ($x \pm$ SD; $n = 3$).

Species	Salinity	K_m (mM)		V_{max}	
	% ASW	P5C	NADH	fmol/cell/min	μmol/mg prot/min
C. autotrophica	50	0.17 ± 0.02	0.09 ± 0.02	0.98 ± 0.05	0.30 ± 0.04
C. autotrophica	150	0.17 ± 0.02	0.10 ± 0.01	4.14 ± 0.19	1.20 ± 0.25
C. saccharophila	50	0.19 ± 0.08	0.10 ± 0.03	8.16 ± 0.06	1.51 ± 0.12
C. saccharophila	150	0.16 ± 0.05	0.10 ± 0.04	10.50 ± 0.90	2.10 ± 0.33

Table II. *Effect of NaCl, KCl, and Proline on the Activity of P5C Reductase from C. autotrophica*

The reaction mixture contained 0.7 mM L-P5C, 0.25 mM NADH, 115 mM NaCl, 20 μL of enzyme, and 50 mM potassium phosphate buffer at pH 7.2, in a total volume of 760 μL. NaCl, KCl, or proline was added to the reaction mixture at the concentrations shown below, and the P5C reductase activity measured for 3 min. Values are averages of duplicate determinations.

Concentration	Activity (% of control)		
M	NaCl	KCl	proline
0.05	100	77	100
0.10	92	68	84
0.20	83	65	64
0.50	55		40
1.00	37	39	26
2.00	29	32	11

Table III. *Effect of Changing the Ratios of NAD^+/NADH or $NADP^+$/NADH on the NADH-Dependent Reduction of P5C by the P5C Reductase of C. autotrophica*

Ratio NAD^+/NADH	Activity	Ratio $NADP^+$/NADH	Activity
	% control		% control
2.0	86	2.0	98
3.5	85	3.5	96
5.5	87	5.5	100
9.2	79	9.2	92
11.0	75	11.0	101
27.6	53	27.6	105
55.0	38	55.0	101

more than 15%, pointing out to the sensitivity of the quaternary structure of the enzyme to its environment. A similar phenomenon has also been observed for P5C reductase from barley seedlings, which showed variations in its estimated mol wt depending on the buffer environment (9). It is not known if such changes in quaternary structure are involved in the regulation of activity of this enzyme *in vivo*.

The kinetics of the reductase were characteristic of a ping-pong mechanism with double competitive substrate inhibition. This mechanism has also been reported for the P5C reductase purified from *Drosophila melanogaster* (5), but it has not previously been reported for plants. Such substrate inhibition is unlikely to be important *in vivo*, where the concentration of P5C and NADH are expected to occur at less than millimolar concentrations but has to be kept in mind when assaying P5C reductase *in vitro*.

In both *Chlorella* species, the P5C reductase showed broad pH optima with little activity above pH 9.0. Even though the reductase showed some dehydrogenase activity at pH 10.4, at physiological pH the enzyme functions as a reductase. It is now believed that the oxidation of proline proceeds via a proline dehydrogenase that does not involve the reduction of $NAD(P)^+$ (3).

The behavior of P5C reductase in *Chlorella* cells grown at different salinities resembles that reported for halophytes (23) and *Pennisetum typhoides* seedlings (7) in that an increase in salinity leads to an increase in P5C reductase activity, especially in salt tolerant cells. In the marine euryhaline *C. autotrophica*, the P5C reductase activity increased 4-fold whereas it only increased 1-5-fold in the terrestrial *C. saccharophila* (Table I). Similarly, a 4-fold increase in P5C reductase activity was observed in the halophyte *M. nodiflorum* in the presence of 400 mM NaCl in contrast to the nonhalophyte *Aster amellus*, which showed an increase of only 1.4 fold in 200 mM NaCl (23). Furthermore, in *M. nodiflorum*, substrate affinity also increased with adaptation to NaCl stress, but this phenomenon was not observed in *Chlorella* (Table I). In a similar way, the presence of only 17 mM NaCl in the growth medium of *Pennisetum typhoides* brought about a 3-fold increase in the activity of P5C reductase (7). In *Nicotiana tabacum*, P5C reductase activity was shown to be directly proportional to proline content from apical to basal leaves (24).

All these facts suggest a role for P5C reductase in the regulation of proline accumulation. However, in *C. autotrophica*, P5C reductase had a maximal velocity of 0.98 fmol/cell/min at 50% ASW (Table I) and the rate of proline synthesis during a transfer from 50 to 150% ASW was equal to 0.023 fmol/cell/min (10). Similarly, the reported values of maximal activities of P5C reductases of *M. nodiflorum* and *N. tabacum* are more than sufficient to account for the observed rates of proline accumulation (23, 24). Under these conditions it is difficult to conceive why the activity of P5C reductase has to increase when the plant is confronted with a salt stress, unless the activity of P5C reductase *in vivo* is much less than the maximal activity reported *in vitro*, which indeed might be the case. First of all, even though the concentration of intracellular P5C is not known, due to its instability at high concentrations and neutral pH (25) and its rate of decomposition proportional to the square of its concentration (12), it

is likely to be in the low micromolar range. In a study of metabolic changes associated with salt adaptation of cell cultures of tomato, the pool of P5C was supposed to be negligible (18) and the intracellular concentration of P5C in fibroblast cells was estimated to be about 20 μM (15). In such conditions, the inhibition of the reductase by NaCl could become significant. It agrees well with the fact that the P5C reductase of *M. nodiflorum* showed 30% inhibition in the presence of 200 mM NaCl and 93 μM P5C, while the inhibition was only 10% with 927 μM P5C. Furthermore, *C. autotrophica* faces the problem of reducing P5C in the presence of high concentrations of proline. Cells transferred from 50 to 150% ASW changed their proline concentration from 160 to 660 mM (10). It is conceivable that as the mass action ratio is shifted toward proline formation, more enzyme is needed to compensate for the inhibition (Table II).

The present study clearly shows that the reduction of P5C in *Chlorella* is specific for NADH as reductant. No inhibition by $NADP^+$ on the NADH-specific reduction of P5C was observed for the *Chlorella* enzyme (Table III), while $NADP^+$ strongly inhibited the P5C reductase from soybean (13). Such high specificity for NADH has not been reported before for P5C reductase of eukaryotes. In higher plants, the situation varies much more. Although the P5C reductase of *M. nodiflorum* was four times more active with NADPH than with NADH (23), reductases from pumpkin (17) and soybean (13) showed the opposite nucleotide specificity. Different nucleotide specificities have also been reported for varieties of the same species; while P5C reductase from *Hordeum vulgare* var Augusta was most active with NADPH (9), the enzyme from *H. vulgare* var Larker preferred NADH over NADPH by a factor of at least threefold (4). Some of this discrepancy could be explained if two P5C reductases were present in plants as has already been suggested (4, 17). However, no isozymes of P5C reductase were found in *C. autotrophica* or *C. saccharophila*. Interestingly, the P5C reductase of salt adapted cells of tobacco exhibited higher affinity for P5C with NADPH than with NADH and the affinity for NADPH increased as the P5C concentration was reduced, leading to the conclusion that NADPH was the preferred substrate (19).

The high specificity shown by the P5C reductase from *Chlorella* for NADH precludes any role for this enzyme in the regeneration of $NADP^+$ required for the synthesis of the purine precursor ribose 5-phosphate as postulated for animal systems and soybean nodules (8). However, the almost complete NADH-specificity of the *Chlorella* enzymes suggests that regulation of NADH concentration may be a mechanism of regulating proline formation. It is generally accepted that in animals and higher plants, the ratio of cytoplasmic NAD^+/NADH is very high (16). In water-stressed bean plants the NAD^+/NADH ratio has been estimated to be close to 500 (14). It is obvious that such high ratios will limit the reduction of P5C in *Chlorella* cells (Table III).

In conclusion, the present data demonstrate that the increase in the maximal velocity of the P5C reductase following a transfer to high salinities was much more pronounced in the salt-tolerant alga *C. autotrophica* than in *C. saccharophila* that does not accumulate high amount of proline for osmoregulation. The reductase from *Chlorella autotrophica* shows kinetics characteristic of a ping-pong mechanism with double competitive substrate inhibition. The high activity of the enzyme demonstrated *in vitro* is unlikely to occur *in vivo* in the presence of low P5C concentration and high proline and salt concentrations encountered during salinity stress. The reductase activity could also be modulated by the ratio NAD^+/NADH because it shows a high specificity for NADH as a cofactor. This high NADH specificity is not known in other eukaryotes and could be unique to the genus *Chlorella*.

Note Added in Proof

We are informed that the affinity for NADPH did not change as the P5C concentration was reduced. The effect reported by Rhodes *et al.* (19) was solely due to the changes in the imidazole concentration used to neutralize the P5C (PC LaRosa, personal communication).

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