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High-temperature damage and acclimation of the photosynthetic apparatus

II. Effect of mono- and divalent cations and pH on the temperature sensitivity of some functional characteristics of chloroplasts isolated from heat-acclimated and non-acclimated bean plants

V. Goltsev[1], I. Yordanov[2], T. Stoyanova[2] and O. Popov[2]

[1] Sofia State University, Biological Faculty, Sofia, and
[2] Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Abstract. The influence of mono- (K^+) and divalent (Mg^{2+}) cations and protons (pH) on the temperature sensitivity of thylakoid membranes was investigated in three groups of young bean plants (control, heat-acclimated and non-acclimated). Thylakoid-membrane function was monitored by second and millisecond delayed fluorescence and 9-aminoacridine fluorescence quenching. It was established that metal ions at investigated concentrations decreased the thermostability of the photosynthetic parameters – an increase of $MgSO_4$ concentration from 0.1 to 20 mM decreased the temperature of their half-inactivation (T50) by 13° C. At the same time the pH dependence of the thermal stability of these parameters showed a maximum at pH 5.5–6.5. The half-inactivation temperatures of those photosynthetic parameters connected with the ability of the thylakoid membrane to form light-induced proton gradients increased by 6–7° C in the heat-acclimated plants compared with the control. It was assumed that the temperature inactivation of photosynthetic electron transfer and the energization of the thylakoid membrane was determined both by the thermoinduced dissociation of the light-harvesting chlorophyll a/b protein complex from PSII, leading to destruction of the excitation energy transfer to the reaction centres, and by the thermal denaturation of the membrane-protein components. The rate of these processes was probably controlled by the size of the negative surface charge and the viscosity of the thylakoid membrane.

Abbreviations: 9-AA = 9-aminoacridine; DF = delayed fluorescence; LHCP = light-harvesting chlorophyll a/b protein complex; PSI (II) = photosystem I (II); T50 = temperature of 50% inhibition of photosynthetic parameter; Tricine = N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl] glycine

Key words: Cation (thermostability) – Fluorescence, delayed – Fluorescence quenching (9-aminoacridine) – Heat acclimation – *Phaseolus* (chloroplast) – Thermoinactivation.

Introduction

The high-temperature sensitivity of the thylakoid membrane and of the photosynthetic reactions connected with it is well documented, but the information regarding the mechanisms determining temperature injury or heat-adaptation of the photosynthetic apparatus is still insufficient. Scientists are unanimous that thermal injury is connected mainly to disturbances in the integrity of the thylakoid membrane (Santarius 1975; Björkman 1975; Berry et al. 1975; Schreiber and Berry 1977). Thermal injury influences the organization of membrane proteins functioning as photosystem II (PSII; Berry and Björkman 1980; Berry and Downton 1982). According to Venediktov and Krivoshejeva (1984), heat denaturation of proteins is the most probable mechanism for inactivation of chloroplasts. It has been shown that the fluidity of the membrane lipids determines to a considerable degree the stability of the protein components at high temperature (Raison and Berry 1979; Raison et al. 1982). The phase transition caused by high temperatures changes the balance of the hydrophylic and hydrophobic interactions among the supracomplex subunits, a process which leads to their dissociation, inhibits their photochemical activity and strongly decreases the effectiveness of excitating-energy transfer from the light-harvesting complex (LHCP) to the reaction centers of PSII (Armond and Hess 1979; Armond et al. 1980).

Another factor associated with the destabilization of the thylakoid membrane could be its surface charge. The raised electrostatic repulsion of the negatively charged protein molecules could lead to undesirably high lateral diffusion on the membrane surface (Barber 1980, 1981) and, as a result, to a loss of functional activity. In addition, at a higher surface-charge density the lipids become more untidy and the temperature of their phase transition decreases (Träuble and Eibl 1974; Sackman 1983). Depending on the density of membrane charges, the chlorophyll-protein complexes of PSI and PSII could either be accidentally distributed in the membrane matrix or divided, thus changing the interaction between the two photosystems (Barber 1981).

Cytoplasmic pH and ion concentration strongly influence the stability of membrane proteins. As a rule, the stability of in-situ proteins depends not only on the interaction forces within their own polypeptide chain, but also on its interaction with other high- or low-molecular-weight compounds. Small alterations of the proton concentration in the neighbourhood of the thylakoid membrane induce considerable changes in the heat sensitivity of the PSII complex (Weis 1982; Venediktov and Krivoshejeva 1984). At higher salt concentrations, pH decrease (7.5–6.0) leads to a significant increase in membrane thermostability – the T50 increases by about 10° C.

According to Alexandrov (1977), proteins are desisive for adaptation to high temperatures. Adaptation probably occurs at the expense of lowering the flexibility of protein molecules as a result of changes in the intramolecular bonds. Different ligands could decrease the conformational mobility of the protein and hence increase its stability (Yordanov et al. 1987).

Under the influence of high temperatures, the lipid composition of photosynthetic membranes undergoes adaptive changes to ensure that their physical functions – microviscosity and permeability to different ions, especially to protons – are maintained as normal (Goldfeld et al. 1980). There exists a threshold maximal fluidity which is compatible with the maintenance of a native membrane structure and function, and is stable after heat acclimation (Raison et al. 1982).

It has been established that at the level of the thylakoid membrane, acclimation is manifest as an increased thermostability of photoinduced proton-gradient formation (Yordanov et al. 1987).

The aim of this paper was to follow the influence of mono- and divalent metal cations and protons (pH) on the thermal sensitivity of the thylakoid membrane, and on the modifying action of temperature acclimation on its thermostability.

Material and methods

Three groups of young bean (*Phaseolus vulgaris* L.) plants – control, heat-acclimated and non-acclimated to high temperatures – were used (Yordanov 1981; Yordanov et al. 1987). The procedure for chloroplast isolation and measuring the functional photosynthetic parameters were described by Yordanov et al. (1987). Chloroplasts were treated at different temperatures for 3 min immediately before measuring the functional activity. The composition of the reaction medium is shown in the figures.

Results

Second (slow) delayed fluorescence. One of the parameters used to characterize the temperature inactivation of the oxygen-evolving system is the second (slow) component of delayed fluorescence (DF; Lurie and Malkin 1975; Venediktov and Krivoshejeva 1983). Figure 1 shows the dependence of this slow component of DF on 3-min-duration temperature treatments at different pHs for chloroplasts from control, heat-acclimated and non-acclimated plants. The temperature at which a given parameter was inactivated to a 50% of its maximum value (T50) was used as a measure for the heat sensitivity of the investigated photosynthetic parameters. The respective T50s are indicated on the figure. There were no appreciable differences at alkaline pH (≈ 8) in the thermostability of the slow component in the different groups of plants, a result which is in agreement with our previous data (Yordanov et al. 1987). In the pH interval from 5.0 to 7.0, however, the temperature stability (T50) of the acclimated plants increased by 4–5° C in comparison with control and non-acclimated plants.

Cation concentrations in the chloroplast suspension medium and their valence influenced the temperature stability of the slow (second) DF component (Fig. 2). An increase in salt concentration decreased the temperature stability in all cases, e.g. a change in the concentration of $MgSO_4$ from 0.1 to 20 mM decreased T50 by 3–4.5° C and a change for KCl from 1 to 100 mM decreased T50 by 5–6.6° C. The differences among the three groups of plants are mainly evident in the pattern of the salt dependency of T50. All the curves had a region of maximum change of T50, and this characteristic change appeared in all of the three groups at different salt concentrations. Moreover, compared with the other groups, this change occurred at lower concentrations in the controls but at the highest concentrations in the non-acclimated plants. Heat-

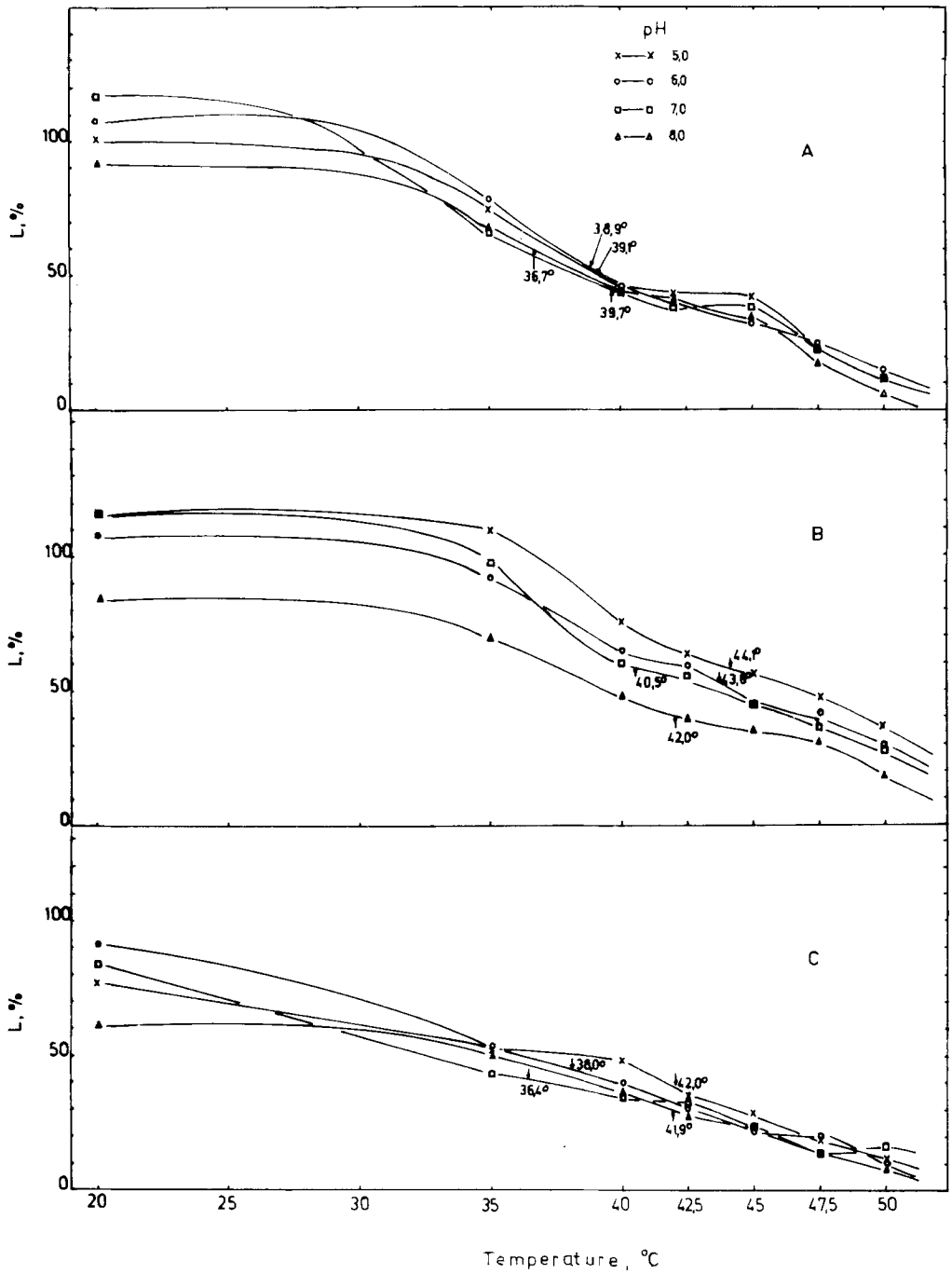

Fig. 1A–C. Temperature inactivation of the second (slow) component of DF of chloroplasts, isolated from control (**A**), heat-acclimated (**B**) and non-acclimated (**C**) bean plants and suspended in media of different pH. Suspending medium contained: 1/15 M phosphate buffer at various pHs, 0.5 M sucrose, 5 mM $MgCl_2$. Chlorophyll concentration = 10 $\mu g \cdot ml^{-1}$

acclimated plants were intermediate in position, although nearer to the controls.

Millisecond delayed fluorescence. In order to evaluate the temperature sensitivity of the photochemical reactions converting the light energy in PSII and the associated energization of the thylakoid membrane, the influence of high temperatures on the induction kinetics of millisecond DF of chloroplasts at different salt content was investigated. Figure 3 shows typical induction kinetics of chloroplasts from heat-acclimated plants suspended in buffer containing 1 or 5 mM $MgSO_4$ and subjected to a 3-min treatment at the indicated temperatures. Temperature treatment led to a general decrease in the intensity of DF and to a change in the induc-

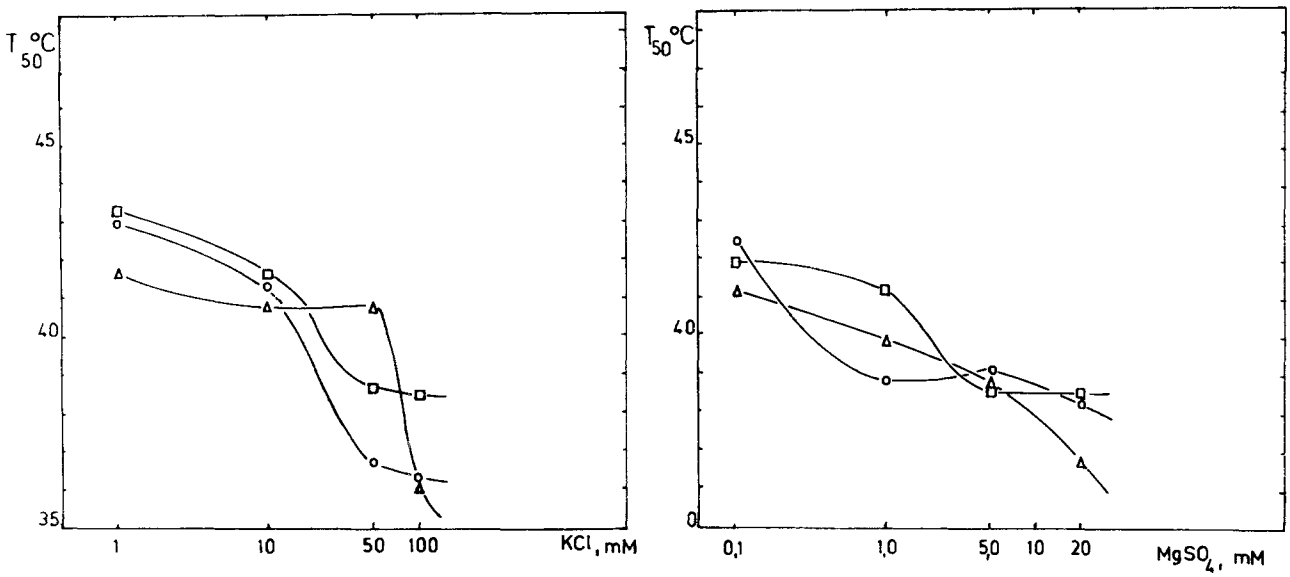

Fig. 2. Dependence of the T50 of the DF second component on concentration of KCl and $MgSO_4$ in the suspending medium of chloroplasts isolated from control (o), heat-acclimated (△) and non-acclimated (□) bean plants. Suspending medium contained: 10 mM Tricine buffer, pH 7.8, 0.65 M sorbitol and various concentrations of mono- or divalent cations. Chlorophyll concentration = 10 $\mu g \cdot ml^{-1}$

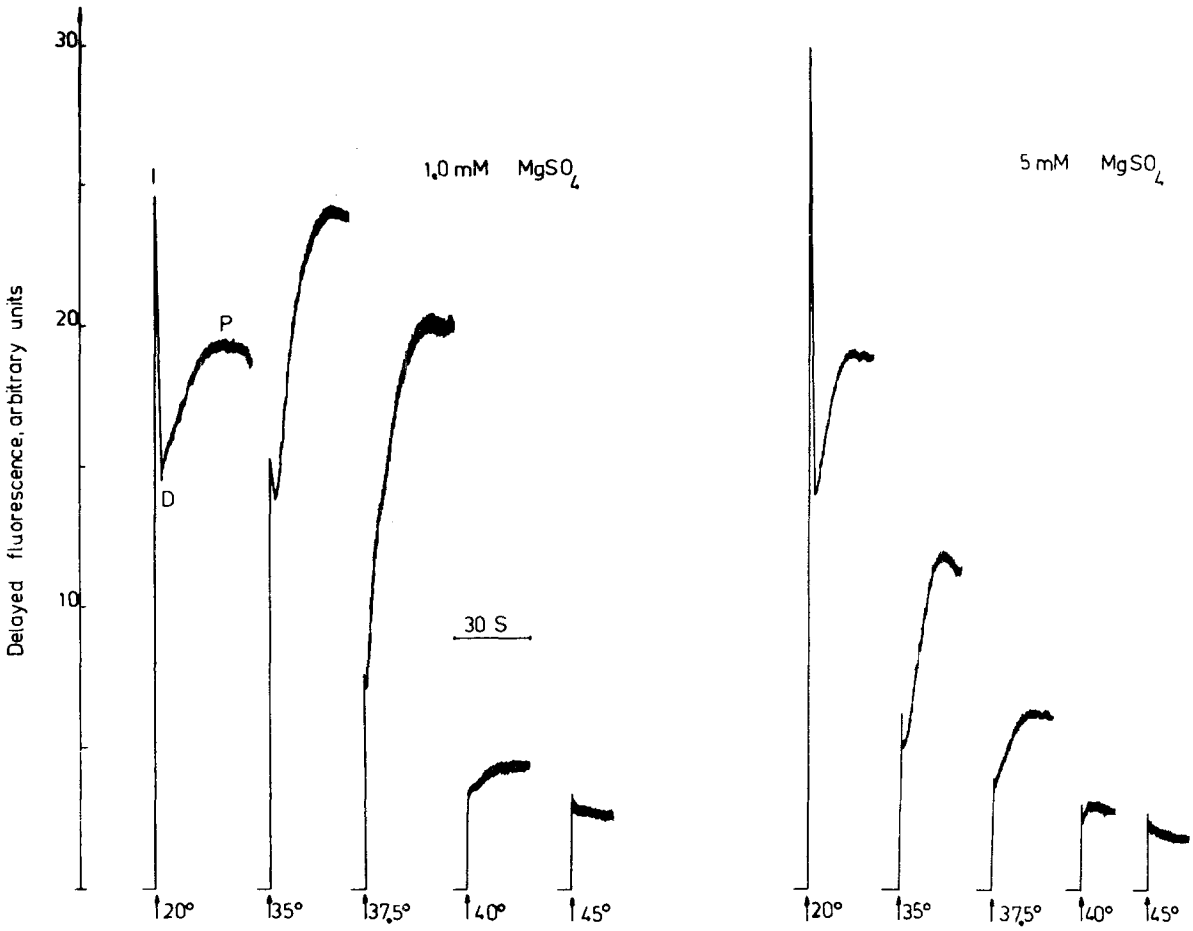

Fig. 3. Induction kinetics of millisecond DF of 1-min-dark-adapted chloroplasts, isolated from heat-acclimated plants, measured after 3 min treatment with the high temperatures indicated under the curves. Chloroplast-suspension medium contained: 10 mM Tricine buffer, pH 7.8, 0.65 M sorbitol and 1 mM or 5 mM $MgSO_4$. Chlorophyll concentration = 10 $\mu g \cdot ml^{-1}$

tion kinetics. Together with the decreases in phases I and D at moderately high temperatures (35–37° C) a rise in phase $D-P$ correlating with the transmembrane ΔpH was observed; this rise is an indication that membrane energization was stimulated under the given conditions. At both concentrations of $MgSO_4$, proton-gradient formation was eliminated after treatment with temperatures higher than 40° C.

The concentration and the valence of cations present in the incubation medium influenced DF induction kinetics and especially the phase $P-D$. Appreciable differences were observed among chloroplasts isolated from the different groups of plants. The absolute DF value for chloroplasts from acclimated plants was about three times higher than that of controls and non-acclimated plants (data not shown). This higher value is probably a consequence of the initially different physiological states of the photosynthetic membranes of chloroplasts isolated from the three groups of plants. For example, there could have been a change in the lability of the bond between the LHCP and the reaction centre of PSII. This was reflected also in the initial differences among the three groups of the photoinduced ΔpH values measured either as DF or as ΔF of 9-aminoacridine (9-AA). Equal DF intensities (the slow component) are an indication that the electron-transfer constants in the reaction centre of PSII were unchanged in the chloroplasts from the three groups of plants.

The pH of the chloroplast suspension medium strongly modified the temperature sensitivity of the two DF induction-curve maxima (I and P, Fig. 4). In practically all cases the maximum thermostability of I and P was observed in the pH range from 5.5 to 6.5. The appreciably lower value of T50 for I of the heat-acclimated chloroplasts compared with the control and non-acclimated ones was striking. It can be assumed that the process of inactivation of the millisecond DF consisted of at least two phases (not shown in the figures) represented by the inactivation curves. One of these phases was obviously connected to the dissociation of LHCP from the reaction centre PSII and the other to changes in the rate constants of electron transfer to reaction centre. Probably, in the control and non-acclimated chloroplasts the contribution by the first phase was negligible, due to some decrease of the energy transfer from LHCP to the reaction centre during isolation. Therefore, the calculated T50 values for control and non-acclimated chloroplasts mainly represented the second, more temperature-stable inactivation phase, whereas for the heat-acclimated chloroplasts the T50 mainly represented the temperature-sensitive phase. For the control and non-acclimated chloroplasts the courses of the pH dependencies of I and P, reflecting the DF quantum yield of the dark and the light-adapted reaction centres, respectively, were similar. With acclimated plants the T50 for phase P of the induction curve at pH 7.0 was higher than for I; this was probably the result of the stabilizing action of the photoinduced transmembrane proton gradient.

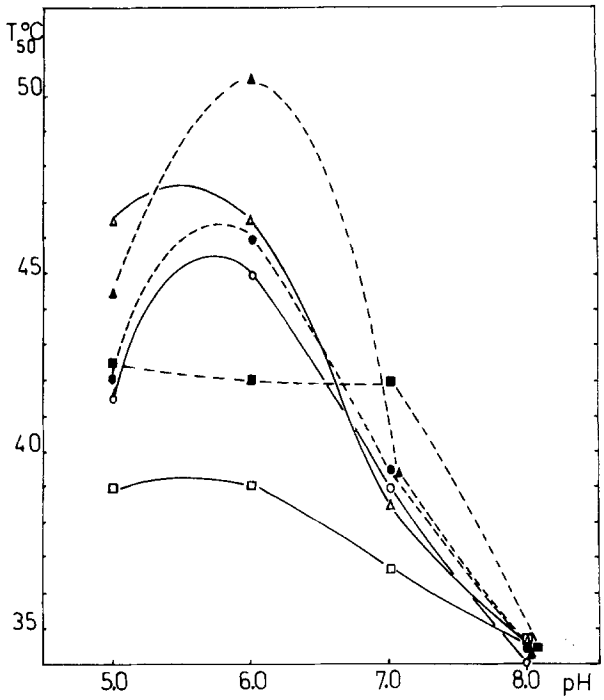

Fig. 4. Dependence of the T50 of the I (○, □, △) and P (●, ■, ▲) phases of DF induction kinetics of chloroplasts isolated from control (○, ●), heat acclimated (□, ■) and non-acclimated (△, ▲) plants on the pH of the suspending medium. Chloroplasts were suspended in 50 mM Tricine buffer with 0.65 M sorbitol and 5 mM $MgSO_4$. Chlorophyll concentration = 10 $\mu g \cdot ml^{-1}$

Temperature-inactivation curves of the maximum DF in phase P for chloroplasts from control, acclimated and non-acclimated plants incubated in the presence of different $MgSO_4$ concentrations are shown in Fig. 5. The thermoresistance, as indicated by the T50 values, was the highest at minimal salt concentration (0.1 mM), and it this case the differences among the three groups of plants were minimal. The differences increased with the rise of Mg^{2+} concentrations to 9° C between the acclimated and control plants at 20 mM $MgSO_4$. Strong enhancement of phase P by Mg^{2+} in the initial part of the temperature curve (to 37° C) was characteristic for the acclimated plants, indicating for a stimulation by high temperatures of the for-

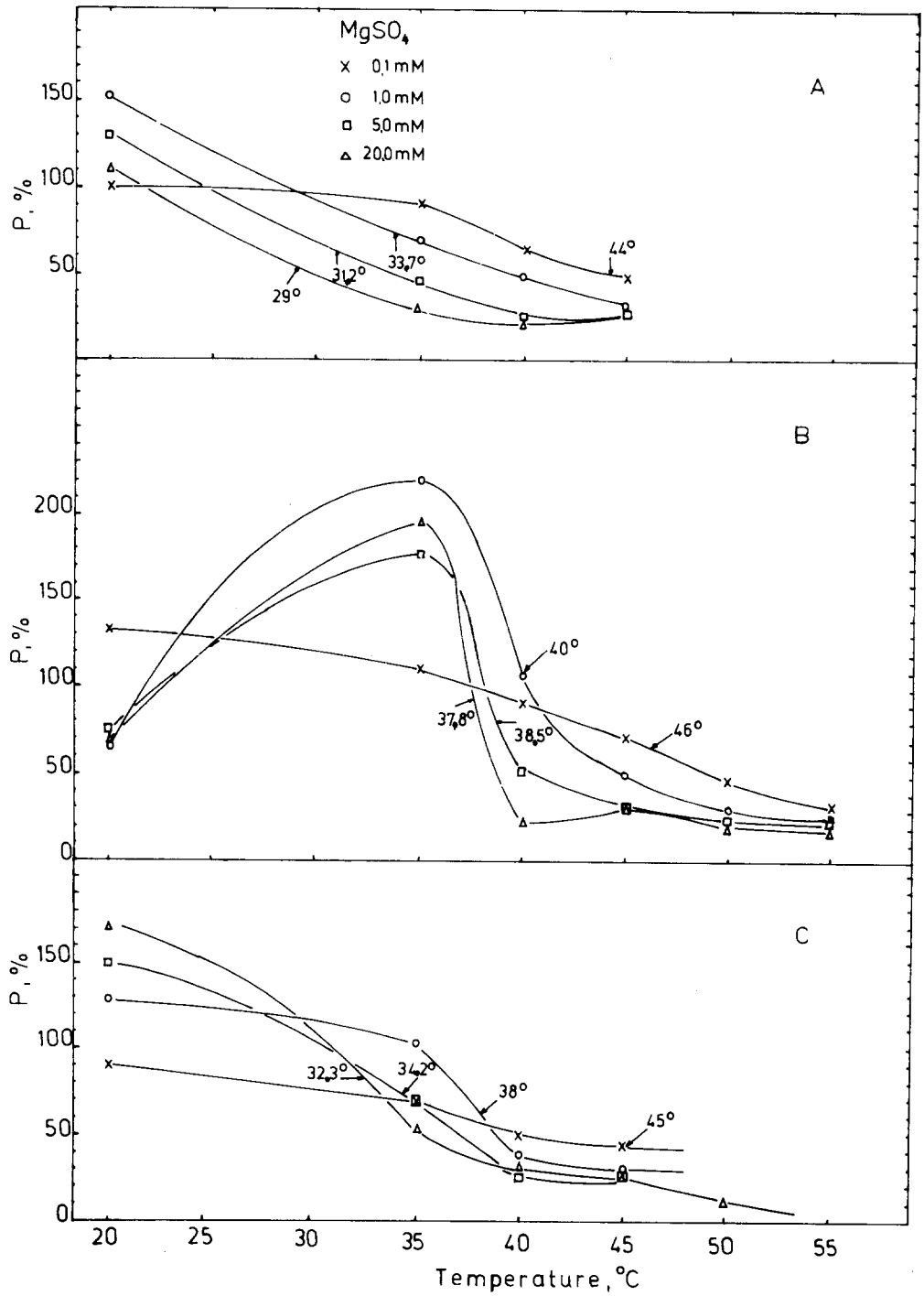

Fig. 5A–C. Dependence of phase P from the induction kinetics of the millisecond DF of chloroplasts isolated from control (**A**), heatacclimated (**B**) and non-acclimated (**C**) plants, on high-temperature preincubation for 3 min. Chloroplasts were suspended in 10 mM Tricine buffer, 0.1 (×), 1.0 (○), 5.0 (□) or 20 (△) mM $MgSO_4$, and sorbitol (final osmotic strength of 0.65 osmol). Chlorophyll concentration = 10 μg · ml^{-1}. The T50 values are indicated by *arrows*

mation of the photoinduced transmembrane proton gradient.

Potassium ions in concentrations an order higher than magnesium destabilized the thermostability in a manner similar to that for the parameters already described (data not shown).

The values of the ratio $\frac{P-D}{D}$, calculated from the induction curves, are correlated with the degree of energization of the thylakoid membrane (ΔpH) and their dependence on temperature is shown in Fig. 6 for the heat-acclimated plants. The ΔpH increased at 20° C with the concentration of Mg^{2+} cations, whereas the T50 decreased from 43.6° C for 0.1 mM to 36.7° C for 20 mM $MgSO_4$. At all salt concentrations there was a strong increase in

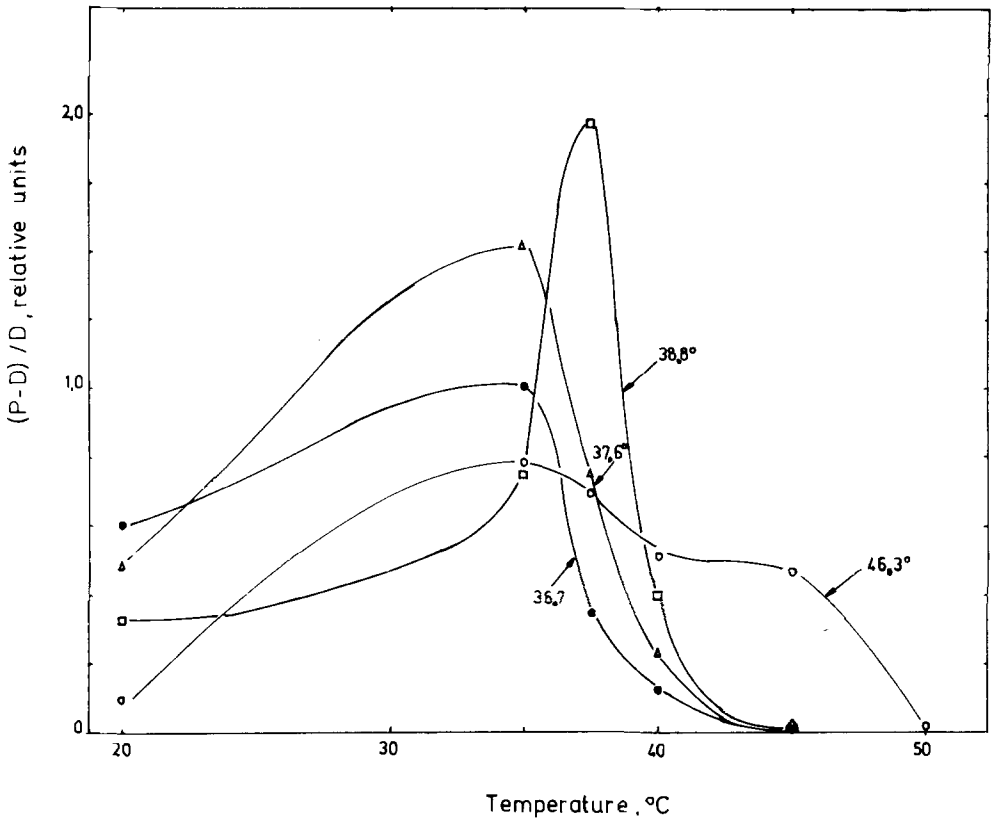

Fig. 6. Influence of a 3-min temperature treatment on the relative change of the millisecond DF $\left(\frac{P-D}{D}\right)$ of chloroplasts, isolated from heat-acclimated plants and suspended in 10 mM Tricine buffer, pH 7.8, with addition of 0.1 (○), 1.0 (□), 5 (△) or 20 (●) mM $MgSO_4$, plus corresponding quantity of sorbitol to a final osmotic strength of 0.65 osmol. The T50 values are indicated by *arrows*

the degree of DF enhancement from the proton gradient as a result of the treatment of isolated chloroplasts with moderately high temperatures upto 35–37° C.

9-aminoacridine fluorescence quenching. Photoinduced quenching of 9-AA fluorescence indicated more directly that the thylakoid membrane is able to generate and to maintain the transmembrane difference in proton concentrations (Schuldiner et al. 1972). The proton gradient in the presence of phenazine methosulfate is a result of a cyclic transfer in *PSI*.

Temperature dependencies of $\frac{F_d - F_l}{F_l}$, directly proportional to $\frac{H^+_{in}}{H^+_{out}}$ are presented in Fig. 7. The chloroplasts from the control and non-acclimated plants generated, at temperature of 20° C, proton gradients about 1–1.3 pH units lower than that of the acclimated plants. The proton gradient decreased uniformly with the increase in the temperature of treatment in the three groups of plants. Temperature acclimation considerably influenced the thermosensitivity of this parameter and the T50 for the acclimated plants was 6–7° C higher than that for the controls and 3.7° C higher than that for the non-acclimated plants.

The temperature sensitivity of ΔpH strongly depended on the salt concentration. Temperature inactivation of fluorescence quenching by the acclimated chloroplasts in the presence of different Mg^{2+} concentrations is shown in Fig. 8. The increase in $MgSO_4$ concentration from 0.1 to 20 mM decreased T50 by 13° C. It was striking that the T50s for ΔpH measured according to this method (Fig. 8) were from 5° C to 13° C (for the different concentrations) higher than those estimated by the ratio $\frac{P-D}{D}$ (Fig. 6). The value of the proton gradient in the first case was determined mainly by the barrier function of the membrane (its permeability to protons) and the rate of cyclic electron transport was probably not limiting. When a proton gradient is formed by noncyclic electron transport, then its temperature sensitivity could be determined either by membrane permeability or by the rate of the electron flow. Since the T50 value for ΔpH obtained by temperature dependencies of the parameter $\frac{P-D}{D}$ (Fig. 6) practically coincided with those of I (data not shown), it can be assumed that the same factor defines the temperature sensitivity of ΔpH and the initial DF intensity (I). This factor is probably the temperature-induced dissociation of the LHCP from the PSII core complex

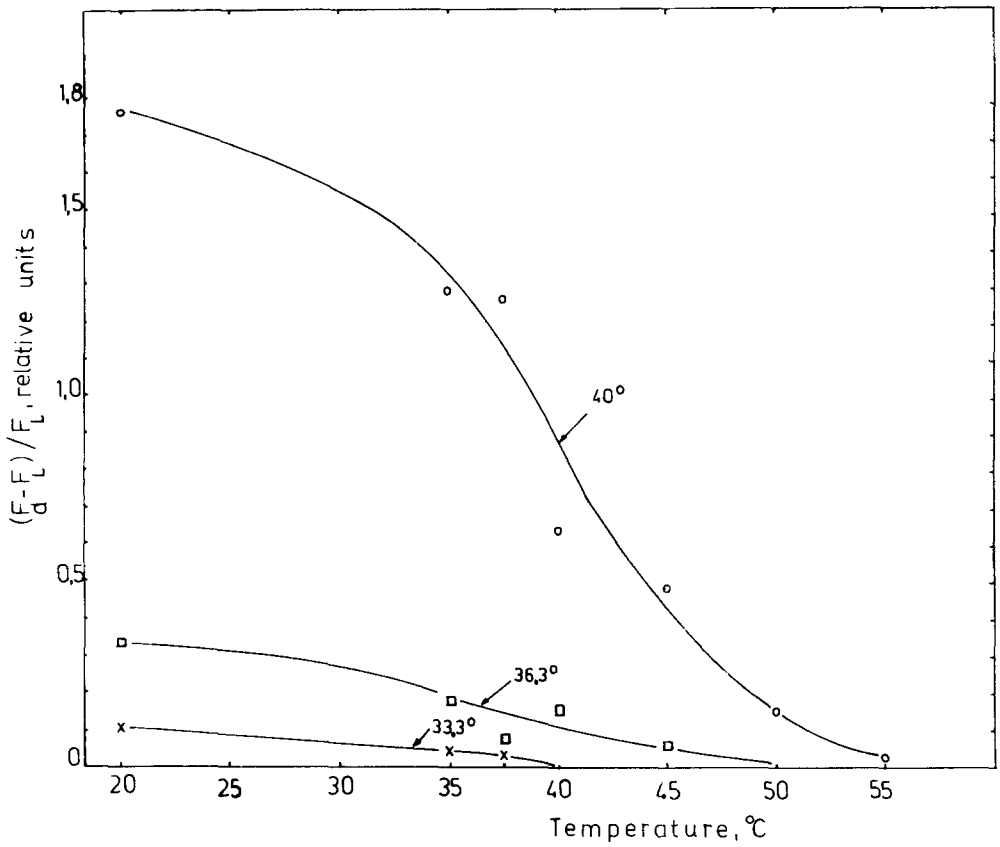

Fig. 7. Influence of a 3-min temperature treatment on the photoinduced 9-AA fluorescence quenching $\left(\frac{F_d-F_l}{F_l}\right)$ by chloroplasts isolated from control (×), heat-acclimated (○) and non-acclimated (□) plants. Chloroplasts were suspended in 10 mM Tricine buffer, pH 7.8, 20 mM $MgSO_4$, 6 μM 9-AA and 100 μM phenazine methosulfate. Chlorophyll concentration = 10 μg·ml^{-1}. The T50 values are indicated by *arrows*

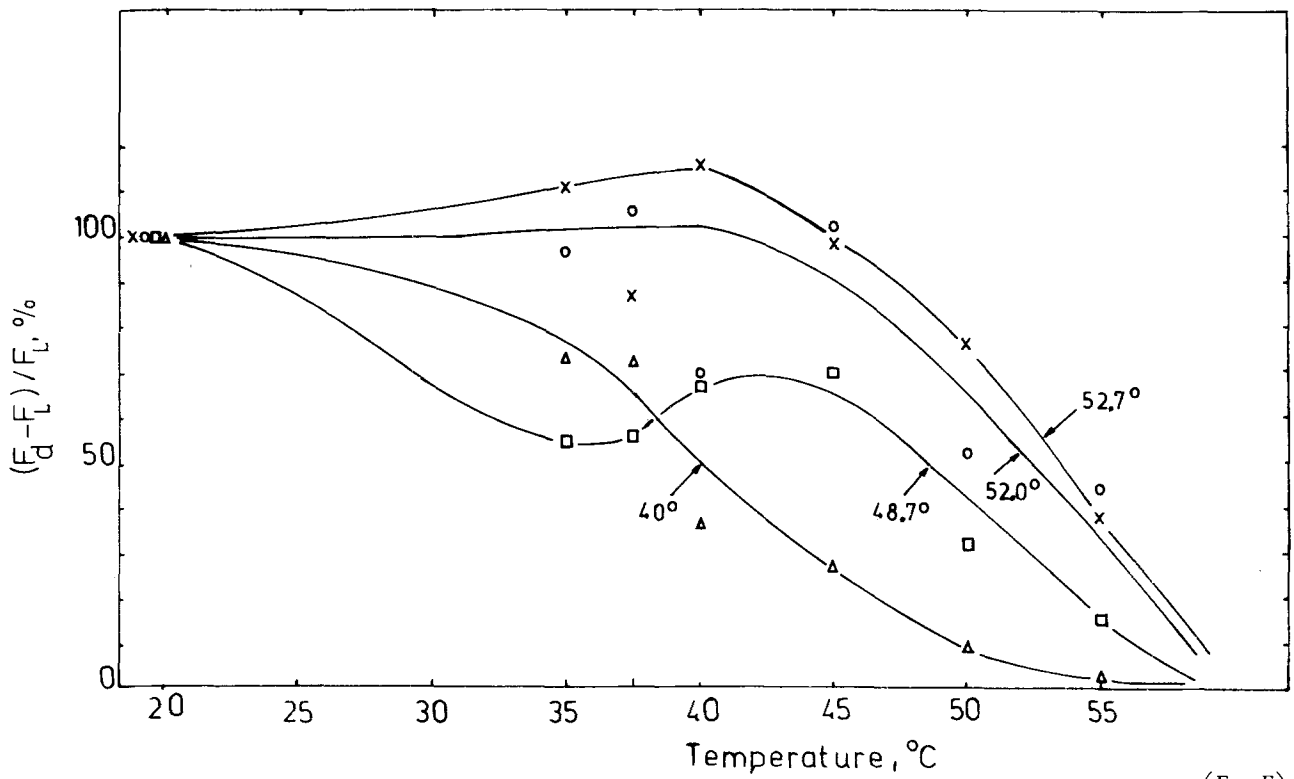

Fig. 8. Influence of a 3-min temperature treatment on the relative changes of photoinduced 9-AA fluorescence quenching $\left(\frac{F_d-F_l}{F_l}\right)$ by chloroplasts isolated from acclimated bean plants and suspended in medium containing: 10 mM Tricine buffer, pH 7.8, 6 μM 9-AA, 100 μM phenazine methosulfate, 0.1 (×), 1.0 (○), 5.0 (□) or 20 (△) mM $MgSO_4$ and sorbitol (final osmotic strength 0.65 osmol). Chlorophyll concentration = 10 μg·ml^{-1}. The T50 values are indicated by *arrows*

and the resulting disturbance in the transfer of exciting energy to the reaction centres.

Discussion

The response of the photosynthetic apparatus to high-temperature action was probably the combination of a great number of partial temperature effects found at different levels of organization. At the level of the thylakoid membrane the high-temperature injury was obviously the result of the denaturation of proteins, or of changes in their conformation. These processes of inactivation occured in, and with the direct participation of, the membrane-lipid physical phase, whose structure and chemical composition determined to a considerable degree the temperature sensitivity of the photosynthetic apparatus as a whole.

The effect of temperature treatment depends on its duration and on the rate of denaturation, which in its turn is defined by the temperature and the proportion of stabilizing and destabilizing factors. Some of these could play a double role: stabilizing in certain cases or destabilizing. The surface and spatial charges of the membrane and membrane lipids could be included among these factors. On the other hand, the molecular charge, originating mainly at the expense of the dissociation of the carboxyl and amino groups, plays a decisive role in the maintenance of the native protein structure in the hydrophylic phase. Hence, the lowering of the total charge of the protein molecule (as a result of the balancing of the positive and negative charge groups or their screening by high concentrations of counter-ions) will decrease its thermostability. On the other hand, the protein molecules built in the membrane could interact through the negative charges (at a physiological pH of the medium) of the ionogen groups displayed on the membrane surface. The result of this interaction would be an increase in the lateral diffusion of molecules or the conformational mobility of single groups leading, in turn, to an acceleration of temperature inactivation. Therefore, the modification of the thylakoid-membrane surface charge caused by changes in the pH or salt composition of the medium is reflected in the temperature stability of the photosynthetic parameters which are sensitive to changes in the native protein conformation. Acidification of the incubation medium from pH 8.0 to 6.0–6.5 will increase the membrane thermostability (as has been shown for a number of photosynthetic parameters) at the expense of a decrease in the total negative charge and the electrostatic – repulsion force among neighbouring proteins. A further acidification of the medium and lowering of the charge will play a decisive role in the decreased stability of the molecules under these conditions. This is reflected in the sharp decrease in the stability at pH 5.0 (Fig. 4), shown also by Weis (1982). A similar phase change in membrane thermosensitivity is to be expected in connection with the dependence of T50 on cation concentrations in the incubation medium. In reality, Weis (1982) showed an increase in the T50 for photosynthetic oxygen evolution following an increase in the ionic strength of the solution at low salt concentrations ($\leq$100 mM KCl). Venediktov and Krivoshejeva (1984) observed an increase in the T50 of the second DF component at low concentrations and a decrease at high concentrations of monovalent ions. Our results showed that T50 decreased for all of the parameters studied in the three groups of plants when both monovalent (K^+, 1–100 mmol·l^{-1}) and divalent (Mg^{2+}, 0.1–20 mmol·l^{-1}) cation concentrations were increased. It is possible that the fundamental differences between our experiments and those of Venediktov and Krivoshejeva are a consequence of the species specificity of the bean thylakoid membrane compared with spinach and pea membranes. Such specificity could be expressed as a decreased density of the surface charge or as a more sparse location of protein molecules on and in the membrane. In both cases, the result would be a lowering of molecular repulsion and a decrease in the role of the surface charge as a destabilizing factor, leading to the predomination of its stabilizing function. In such chloroplast preparations, lowering the surface charge by the addition of salts would be expected to lead to a decrease in the T50. As a rule, the curves of T50 values for the investigated photosynthetic parameters versus salt concentration showed a region of sharp decrease in temperature stability. This decrease occurred at different concentrations in the three groups of plants – control, heat-acclimated and non-acclimated. It can be assumed that at these concentrations the surface-charge value is such that a decrease would be critical for the rate of temperature-induced denaturation. The specific concentrations for the three groups of plants were different, and this is an indication of differences in the surface structure of the membranes (possibly there are different distributions of the protein molecules).

An alternative or possibly an additional explanation for the ability of salts to lower the temperature stability of the photosynthetic parameters could be the presence and the proportions of the two forms of LHCP, one form tightly associated

with PSII and the other form structurally free (Haworth et al. 1982). Obviously the temperature at which a dissociation of the supracomplex could occur would be different for each LHCP form – the T50 would be lower for the structurally free form. Assuming that both LHCP forms carried a different total negative charge, the curve for the temperature inactivation of a given photosynthetic parameter would not be sigmoidal but more complex (step-like form) reflecting the superimposition of two inactivation processes with different characteristic temperatures. We obtained exactly that type of inactivation curve for the millisecond DF components whose values at unsaturated intensities of actinic light were proportional to the effectiveness of the excitation-energy transfer from LHCP to the reaction centre of PSII.

At physiological pHs of the chloroplast-suspension medium the ionic concentration controls the degree of grana stacking of the thylakoid membranes. At low ionic concentrations the thylakoids swell, the distance between them increases and the area of appressed region decreases. In addition, part of the LHCP, mainly the structurally free form, dissociates from the respective reaction centre and becomes redistributed in the membranes of the stromal thylakoids (Barber 1984). As a result the chloroplast photosynthetic membranes suspended in media with different ion compositions would have different proportions of their supracomplexes built from tightly associated and free LHCP. In chloroplasts with tightly stacked granal thylakoids, as would be found in a high-ionic-strength medium, almost all the LHCP of both forms would be associated with the reaction centres. The curve of temperature inactivation of some photosynthetic parameters in such chloroplasts would have a biphasic character reflecting the different temperature sensitivity of the free- and tightly bound LHCP. On the other hand, in low-ionic-strength media when only the reaction centres associated with the tightly bound LHCP are photosynthetically active, the character of the T50 dependence of the separate photosynthetic parameters on the salt concentrations in the chloroplast-suspension medium would depend on the ratio of the two LHCP in the supracomplexes of the photosynthetic apparatus. If the photosynthetic apparatus is represented mainly by tightly bound LHCP, salts would increase the temperature stability of the chloroplasts, but if loosely connected LHCP predominated, the salt concentration would lead to a lowering of the stability. It is possible that the differences between the results obtained in this work and the other published data, mentioned above, are related to the different proportions of tightly and loosely bound LHCP in the photosynthetic apparatus of the investigated plants.

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