

Mitochondrial Adaptations to NaCl. Complex I Is Protected by Anti-Oxidants and Small Heat Shock Proteins, Whereas Complex II Is Protected by Proline and Betaine¹

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High soil sodium (Na) is a common stress in natural and agricultural systems. Roots are usually the first tissues exposed to Na stress and Na stress-related impairment of mitochondrial function is likely to be particularly important in roots. However, neither the effects of NaCl on mitochondrial function, nor its protection by several potential adaptive mechanisms, have been well studied. This study investigated the effects of NaCl stress on maize (*Zea mays*) mitochondrial electron transport and its relative protection by osmoprotectants (proline, betaine, and sucrose), antioxidants (ascorbate, glutathione, and α -tocopherol), antioxidant enzymes (catalase and Cu/Zn-superoxide dismutase), and mitochondrial small heat shock proteins (sHsps). We demonstrate that Complex I electron transport is protected by antioxidants and sHsps, but not osmoprotectants, whereas Complex II is protected only by low concentrations of proline and betaine. These results indicate that NaCl stress damaged Complex I via oxidative stress and suggests that sHsps may protect Complex I as antioxidants, but NaCl damaged Complex II directly. This is the first study to demonstrate that NaCl stress differentially affects Complex I and II in plants and that protection of Complex I and II during NaCl stress is achieved by different mechanisms.

Diverse environmental stresses often induce similar kinds of cellular damage. For example, many, or even most, environmental stresses induce oxidative stress and protein denaturation (e.g. temperature stress, salinity, and drought; Vierling, 1991; Bowler et al., 1992; Parsell and Lindquist, 1994; Navari-Izzo et al., 1996; Waters et al., 1996; Noctor and Foyer, 1998). As a consequence, diverse stresses often illicit similar cellular adaptive responses, such as the production of stress proteins, up-regulation of oxidative stress protectors, and accumulation of protective solutes (e.g. Vierling, 1991; Bowler et al., 1992; Parsell and Lindquist, 1994; Navari-Izzo et al., 1996; Hare et al., 1998; Noctor and Foyer, 1998; McNeil et al., 1999; Hamilton et al., 2001). In many cases, mitochondria are key sites of damage during environmental stress, especially mitochondrial electron transport (e.g. Chauveau et al., 1978; Zhang et al., 1990; Hernandez et al., 1993; Polla et al., 1996; Pobezhimova et al., 1997; Yan et al., 1997; Downs and Heckathorn, 1998). Most of the general cellular protective adaptations mentioned above are known to be present in mitochondria (e.g. Vierling, 1991; Parsell and Lindquist, 1994; Prasad et al., 1995; Waters et al., 1996; Jimenez et al., 1997, 1998; Downs and Heckathorn, 1998); however, the relative importance of these adaptations is unknown. We predict that the relative importance of these adaptations will vary among stresses,

because the specific nature of "damage" varies with the type of stress. For example, there are "weak links" within mitochondria during stress and these weak links are different for different stresses (Zhang et al., 1990; Hernandez et al., 1993; Polla et al., 1996; Pobezhimova et al., 1997; Yan et al., 1997; Downs and Heckathorn, 1998). Also, it is unlikely that any one specific adaptation can protect all weak links.

A widespread and common stress in natural and agricultural systems is soil sodium (Na) stress, which influences plant distributions and can significantly reduce plant productivity (Boyer, 1982). Roots are usually the first tissues exposed to Na stress. For this reason, and the fact that roots depend on mitochondria for most of their cellular energy production, Na stress-related impairment of mitochondrial function is likely to be particularly important in roots. Yet, relatively little work has been done on the responses of mitochondria to Na stress (Livne and Levin, 1967; Jolivet et al., 1990; Hernandez et al., 1993). For example, despite the extensive research conducted on plant responses to salinity, it is not known in detail how salinity affects mitochondrial respiration.

In this study, we analyzed the effects of Na stress on Complex I and II of the electron transport chain. We then determined the efficacy of protection of electron transport during Na stress by three osmoprotectants (Pro, betaine, and Suc), three antioxidants (ascorbate, glutathione, and α -tocopherol), two antioxidant enzymes (catalase and Cu/Zn-superoxide dismutase [SOD]), and purified small heat shock proteins (sHsps; i.e. mitochondrial sHsp). The osmoprotectants of interest potentially act in two ways: (a)

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through balancing osmotic potential (Ladyman et al., 1983; Hare et al., 1998; McNeil et al., 1999), and (b) by direct stabilization of membranes and/or proteins (Paleg et al., 1984; Lee et al., 1997; Rajendrakumar et al., 1997; Hare et al., 1998; McNeil et al., 1999). The antioxidants and enzymes above all act to scavenge oxygen radicals and their byproducts (e.g. O_2^- , H_2O_2 , and OH^\cdot); collectively they are termed active oxygen species (AOS). Ascorbate and glutathione are involved in scavenging primarily via the Halliwell-Asada pathway as substrates for ascorbate peroxidase (APX), which scavenges H_2O_2 and glutathione reductase that is involved in the regeneration of ascorbate (Noctor and Foyer, 1998; Asada, 1999). α -Tocopherol directly scavenges singlet oxygen and peroxides and can be regenerated by ascorbate and associated enzymes of the Halliwell-Asada pathway (Hess, 1993). SOD detoxifies superoxide to H_2O_2 (Bowler et al., 1992) and catalase detoxifies H_2O_2 to water and oxygen (Prasad et al., 1995). The small Hsps protect electron transport during heat and oxidative stress (e.g. oxidative stress resulting from exogenous H_2O_2) in mitochondria and during heat, oxidative stress, and photoinhibition in chloroplasts (Downs and Heckathorn, 1998; Heckathorn et al., 1998; Downs et al., 1999a, 1999b). We demonstrate that Complex I electron transport was protected by antioxidants and sHsps, but not by osmoprotectants. The results indicate that Na stress damaged Complex I via oxidative stress and suggests that sHsps may protect Complex I as site-specific antioxidants. Complex II was protected by low concentrations of osmoprotectants. Therefore, Na stress apparently damaged Complex II directly.

RESULTS

To determine the relative efficiency with which certain adaptive proteins and metabolites protect mi-

tochondrial electron transport during Na stress, we added each protective component to Na-stressed sub-mitochondrial particles and measured electron transport either from (a) Complex I (NADH:ubiquinone oxidoreductase) to Complex III (ubiquinol:cytochrome *c* oxidoreductase) to Complex IV (cytochrome *c* oxidase) to O_2 , or (b) Complex II (succinate:ubiquinone oxidoreductase) to Complex III and IV to O_2 . For simplicity, and because differences in electron transport between Complex I to IV versus Complex II to IV will reflect differences between Complex I and II, we refer to electron transport from I to IV as Complex I and electron transport from II to IV as Complex II. Salicylhydroxamic acid (SHAM) was added to all assays as an inhibitor of the alternative oxidase pathway to measure electron flow only through the desired complexes. When measuring Complex I electron flow, Complex II was inhibited by adding thenoyltrifluoroacetone and rotenone was added to inhibit Complex I while measuring Complex II.

NaCl Effects on Electron Transport

NaCl stress reduced the rate of Complex I electron transport by 22% at 50 mM and by >50% for all other treatment levels (Fig. 1A). The rate of Complex II was reduced by 32% at 50 mM and >45% for all other treatments (Fig. 1B). Electron transport was not further reduced by 200 mM NaCl compared with 100 mM NaCl; therefore, the 100-mM treatment was used for subsequent protection assays.

Osmoprotectants

Complex I electron transport was not protected from NaCl stress by Pro, betaine, or Suc at any concentration of the osmoprotectants assayed (Fig. 2,

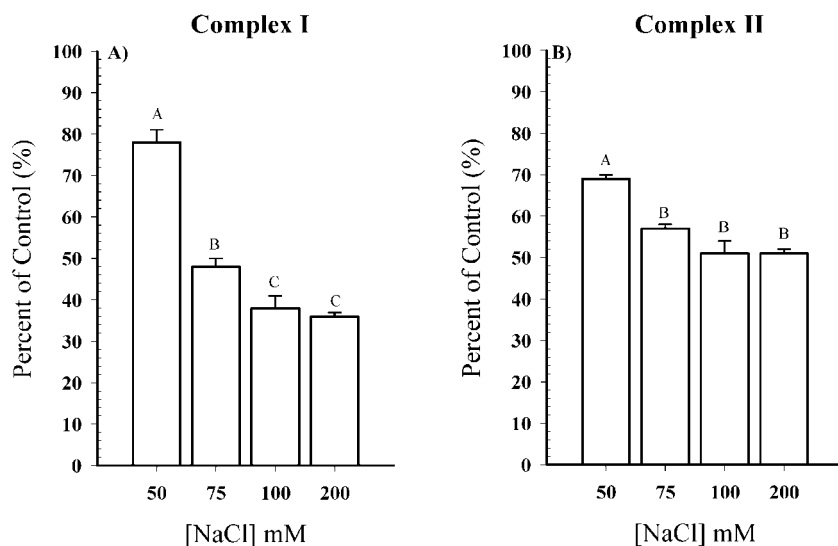
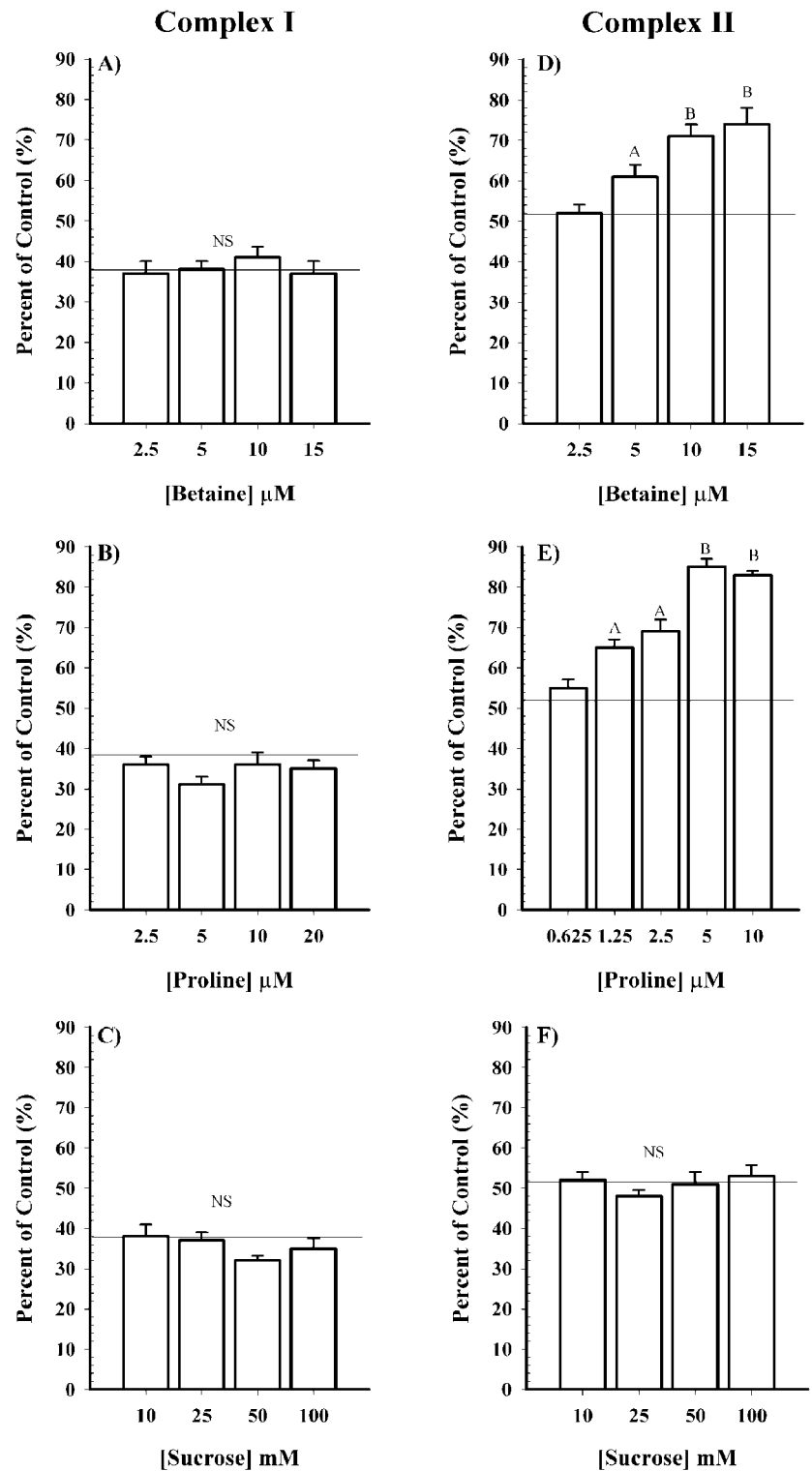


Figure 1. Disruption of Complex I (A) and Complex II (B) electron transport (defined in "Results") in sub-mitochondrial particles as a percent of control rates prior to NaCl treatment. NaCl was added after the establishment of a linear control rate, which was 310 ± 6 nmol O_2 mg^{-1} protein min^{-1} for Complex I and 245 ± 8 nmol O_2 mg^{-1} protein min^{-1} for Complex II. Results are means \pm 1 SE; $n = 12$ (three each from four preparations of sub-mitochondrial particles). ANOVAs were performed for each complex and bars with different letters are significantly different at $P < 0.05$ from a Tukey's post-hoc means comparison.

Figure 2. A–C, Complex I; D–F, Complex II electron transport (defined in “Results”) in sub-mitochondrial particles as a percent of control rates when betaine, Pro, and Suc were added after the establishment of a linear control rate but prior to NaCl addition (See Fig. 1 for rates). Results are means \pm 1 SE; $n = 6$ (three each from two preparations of sub-mitochondrial particles). ANOVAs were performed with Tukey’s post-hoc means comparisons for each complex and bars with different letters are significantly different at $P < 0.05$ and bars marked NS are not significantly different. The horizontal lines represent the mean of the 100 mM NaCl treatment without any protectants, obtained from Figure 1, for either Complex I or II.



A–C). In contrast, Complex II electron transport was protected by betaine and Pro, but not by Suc (Fig. 2, D–F). Protection by betaine was titratable and was maximal between 10 and 15 μ M, which provided a 20% higher rate of electron transport when compared with NaCl stress. Protection by Pro was also titrat-

able and was maximal between 5 and 10 μ M, which provided a 30% higher rate of electron transport. The addition of Trp (a negative control for amino acids) did not protect either Complex I or II electron transport (data not shown). The protection of Complex II electron transport by betaine and Pro resulted from

the direct protection of Complex II and not via a change in osmotic potential because the concentrations of Pro and betaine in the assays were four orders of magnitude lower than the total osmotic concentration of the assay medium (containing 320 mM mannitol). Suc, when added at millimolar concentrations, did not protect Complex II, which is further support that protection by betaine and Pro resulted from direct interaction with Complex II, rather than by osmotic effects.

Antioxidants

Complex I electron transport was protected by all three antioxidant metabolites assayed (Fig. 3, A–C), but to varying degrees. In contrast, Complex II electron transport was not protected at any concentrations of the antioxidants assayed (Fig. 3, D–F). α -Tocopherol protected Complex I at 20 mM (20% above NaCl treatment) and protection did not increase at 30 mM. This suggests that the damage to

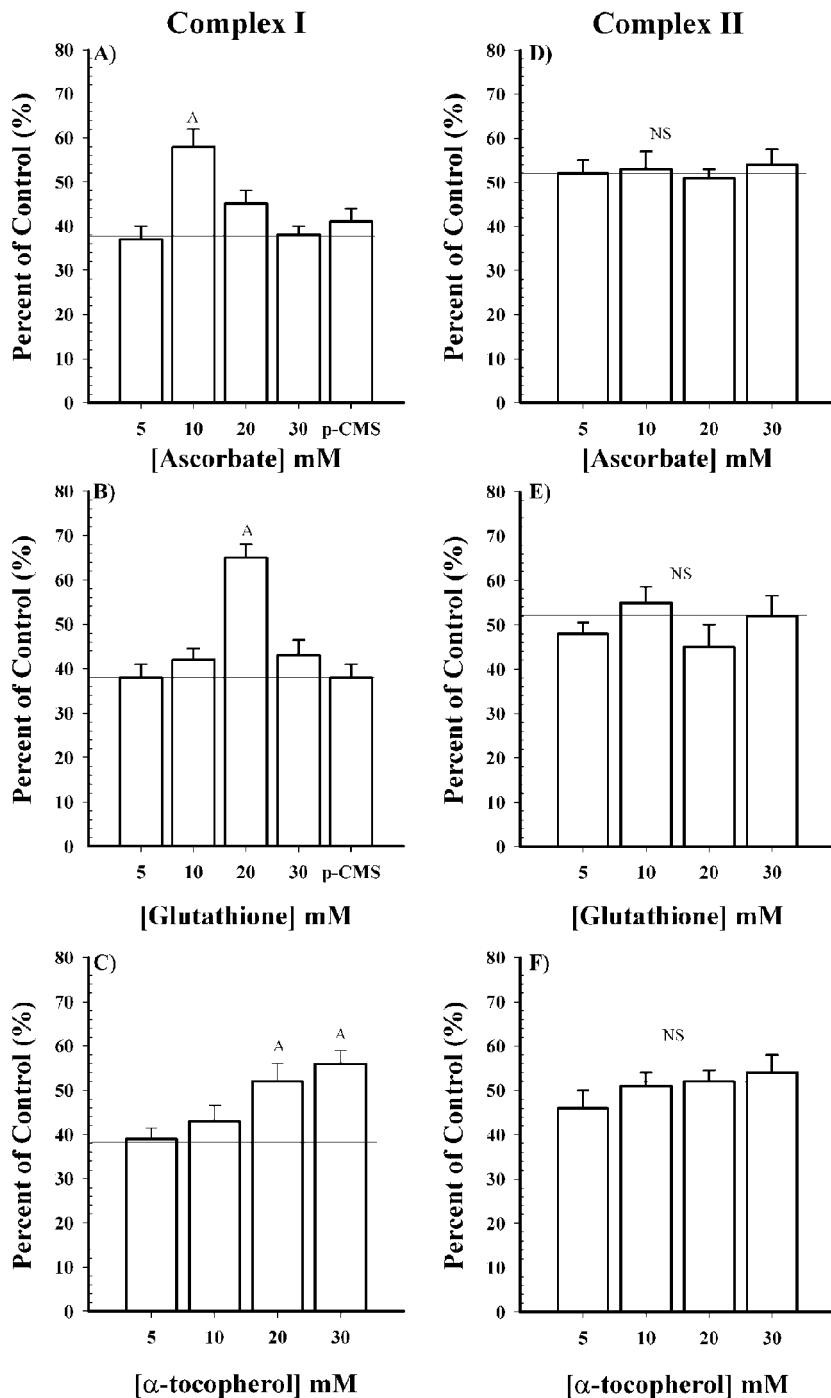


Figure 3. A–C, Complex I; D–F, Complex II electron transport (defined in “Results”) in sub-mitochondrial particles as a percent of control rates when ascorbate, glutathione, and α -tocopherol were added after the establishment of a linear control rate but prior to NaCl addition (see Fig. 1 for rates). Results are means \pm 1 SE; $n = 6$ (three each from two preparations of sub-mitochondrial particles). ANOVAs were performed with Tukey’s post-hoc means comparisons for each complex and bars with different letters are significantly different at $P < 0.05$ and bars NS are not significantly different. The horizontal lines represent the mean of the 100 mM NaCl treatment without any protectants, obtained from Figure 1, for either Complex I or II.

Complex I is the result of oxidative stress. Ascorbate and glutathione were equally effective at protecting electron transport, but at different concentrations. Ascorbate protected Complex I only at the 10-mM level and protection by glutathione was observed only at 20 mM (Fig. 3, A and B). It is important that protection of Complex I by ascorbate and glutathione did not occur at higher concentrations. The addition of p-chloromercuribenzoic acid (p-CMB), an inhibitor of APX (Mittler and Zilinskas, 1993), prior to or after NaCl treatment, to either the ascorbate or glutathione protection assay, prevented protection of Complex I (Fig. 3, A and B). Thus, ascorbate and glutathione did not protect Complex I directly. This result was confirmed by the addition of 10 mM dithiothreitol (DTT; data not shown), which inhibits APX in the presence of peroxide (Chen and Asada, 1992). This identifies a role for the Halliwell-Asada pathway in protecting mitochondria exposed to NaCl stress and suggests that detoxification of H_2O_2 by APX is important in the adaptation of mitochondria to oxidative stress.

Enzymes and Hsps

Complex I electron transport was protected by SOD, catalase, and purified mitochondrial sHsps (Fig. 4, A–C), and this protection was titratable. On average, they increased Complex I electron transport by 33% above NaCl stressed rates. The protection of electron transport by the mitochondrial sHsps was inhibited by the addition of an antibody that is specific to these sHsps (Fig. 4C), which disrupted the interaction of sHsp with Complex I (as in Heckathorn et al., 1998; Downs and Heckathorn, 1998). The addition of pre-immune serum had no effect on protection (Fig. 4C). These results suggest that NaCl stress is damaging Complex I electron transport primarily through oxidative stress and that mitochondrial sHsps may be acting as antioxidants. In contrast to Complex I, Complex II electron transport was not protected by either SOD, catalase, or mitochondrial sHsps (Fig. 4, D–F).

DISCUSSION

To our knowledge, this is the first study to demonstrate that: (a) Na stress differentially affects Complex I and II in plants; (b) protection of Complex I and II during Na stress by several well-known mechanisms occurs; however, (c) anti-oxidant enzymes, as well as small Hsps, protect Complex I, but not Complex II, whereas the osmoprotectants, Pro and betaine, protect Complex II, but not Complex I. These results suggest that Complex I is disrupted by Na as a result of oxidative stress and that Complex II was disrupted by Na toxicity. Further, protection of Complex I was greatest for SOD, suggesting that the basal cause of oxidative damage in Complex I is generation of $O_2^{\cdot-}$.

Analysis of the protective effect of the antioxidants glutathione and ascorbate indicated that these metabolites do not protect Complex I directly, but via the Halliwell-Asada pathway. The addition of inhibitors (p-CMB and DTT), which are thiol reagents that inhibit APX, prevented protection by either ascorbate or glutathione, but when these inhibitors were added in the absence of NaCl, there was no effect on electron transport (data not shown). Therefore, there is strong evidence that the Halliwell-Asada pathway is functional in mitochondria during NaCl stress and is effective in protecting Complex I from NaCl-induced oxidative stress. The concentrations at which ascorbate and glutathione protected Complex I are physiologically relevant when compared with measurements from other studies (Noctor and Foyer, 1998). It is interesting that protection was lost for both ascorbate and glutathione at concentrations only 10 mM higher than the levels that provided protection. Increased production of glutathione actually increased oxidative stress in transgenic tobacco (Creissen et al., 1999), a result that supports the findings in this study. This may be due to a change in the redox potential of the media and or direct inhibition of Complex I at high concentrations. This suggests that tight control of ascorbate and glutathione levels are necessary for the Halliwell-Asada pathway to be effective in protecting Complex I electron transport.

The ability of the AOS scavenger enzymes, SOD and catalase, to protect Complex I activity provides another line of evidence that demonstrates that NaCl disrupts activity via oxidative stress. Although they were capable of significant protection, neither enzyme could entirely prevent damage, which may be due to the fact that they detoxify different AOS. One might expect that the addition of both SOD and catalase would provide greater protection of electron transport than either added singly, given that SOD decreases $O_2^{\cdot-}$ and catalase decreases H_2O_2 and $O_2^{\cdot-}$, and H_2O_2 reacts to produce the most toxic AOS known, OH^{\cdot} . We did not investigate this possibility, and it is not known if mitochondrial SOD and catalase activities both increase during salt stress. In salt-stressed pea mitochondria, Mn-SOD activity is increased and is correlated with salt tolerance of specific genotypes (Hernandez et al., 1993), and although a mitochondrial-localized catalase has been characterized in maize (*Zea mays*; Scandalios et al., 1980, 1984; Prasad et al., 1995), it is not known if activity of this catalase also increases during salt stress.

In addition to protection of Complex I by antioxidant enzymes, we observed protection of Complex I by α -tocopherol. α -Tocopherol directly scavenges $O_2^{\cdot-}$ to produce α -tocopheroxyl, which can be converted to α -tocopherol by ascorbate, to yield monodehydroascorbate (Hess, 1993). The location of α -tocopherol in membranes (Hess, 1993) makes it an effective constitutive scavenger of $O_2^{\cdot-}$, although it

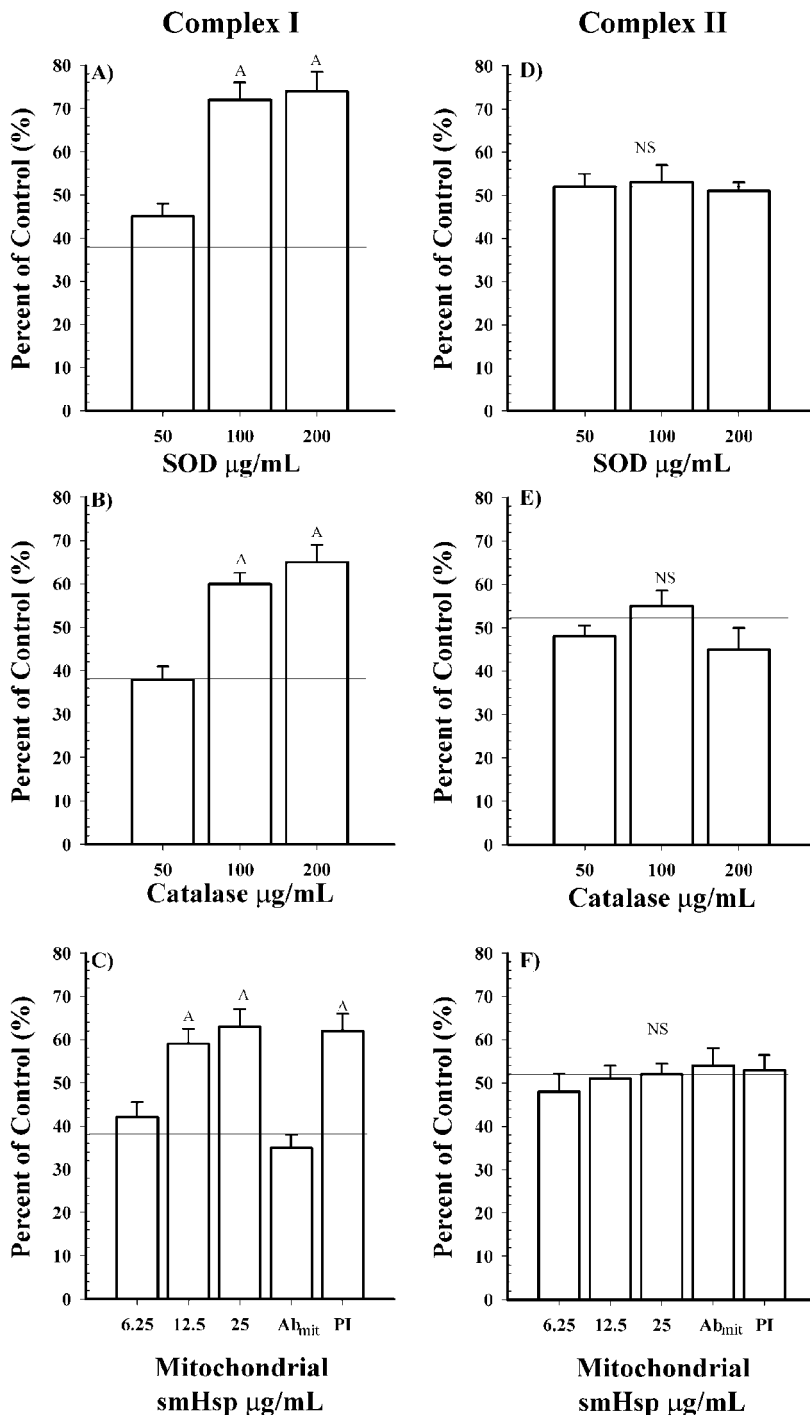


Figure 4. A–C, Complex I; D–F, Complex II electron transport (defined in “Results”) in sub-mitochondrial particles as a percent of control rates when SOD, catalase, and purified mitochondrial sHsps were added after the establishment of a linear control rate but prior to NaCl addition (See Fig. 1 for rates). Results are means \pm 1 SE; $n = 6$ (three each from two preparations of sub-mitochondrial particles). ANOVAs were performed with Tukey’s post-hoc means comparisons for each complex and bars with different letters are significantly different at $P < 0.05$ and bars NS are not significantly different. The horizontal lines represent the mean of the 100 mM NaCl treatment without any protectants, obtained from Figure 1, for either Complex I or II.

would only be effective if it was regenerated rapidly. α -Tocopherol also directly stabilizes membranes (Hess, 1993). There is correlative data from several studies (Hendry and Brocklebank, 1987; Price and Hendry, 1989; Anderson et al., 1992) that shows that environmental stress influences tissue levels of α -tocopherol and that its concentration is correlated with tolerance (Price and Hendry, 1989).

It is interesting that mitochondrial sHsps protected Complex I during Na stress, but not Complex II.

Because Complex I was protected only by antioxidants, and Complex II only by osmoprotectants, this suggests the possibility that mitochondrial sHsps protect Complex I through an anti-oxidant mechanism. There is already some evidence to suggest that chloroplast sHsps protect photosynthetic electron transport in this way (Downs et al., 1999b). To explain, chloroplast sHsps contain a “Met-rich” domain, and this domain is partially conserved in mitochondrial sHsps (Lenne et al., 1995; Waters et al.,

1996). There is accumulating evidence that Met residues in some proteins may function as anti-oxidants, via oxidation to Met sulfoxide (Levine et al., 1996; Moskovitz et al., 1997). We have obtained preliminary (unpublished) results, from x-ray absorption spectroscopy of purified chloroplast sHsp, that the Met residues in the chloroplast sHsps are oxidized during heat stress, and Härndahl et al. (1999) have shown that purified chloroplast sHsps are reversibly oxidized and reduced in vitro. In addition, chloroplast sHsps protect photosynthetic electron transport in vitro from oxidative damage resulting from exogenous H₂O₂ (Downs et al., 1999). Perhaps mitochondrial sHsps protect electron transport by a mechanism that is similar to that of the chloroplast sHsps, and that this mechanism involves AOS scavenging by Met residues.

The results from the protection of Complex II by Pro and betaine are less conclusive with respect to the type of damage that reduced electron transport. However, the data suggest that the reduction in Complex II was not the result of oxidative stress because none of the antioxidants assayed provided protection. In this study, the concentrations of Pro and betaine were not high enough to act as osmotica (Ladyman et al., 1983; Hare et al., 1998; McNeil et al., 1999). In other systems, betaine and Pro directly stabilize membranes and proteins (Paleg et al., 1984; Lee et al., 1997; Hare et al., 1998; McNeil et al., 1999).

MATERIALS AND METHODS

Sub-mitochondrial Particle Preparation

Sub-mitochondrial particles were prepared from maize (*Zea mays*) using a method modified from Douce et al. (1987) and Lund et al. (1998). Hypocotyls from etiolated 7-d-old seedlings were homogenized in a mitochondrial isolation buffer (MIB) containing 350 mM mannitol, 30 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] (pH 7.6), 1 mM EDTA, 4 mM L-Cys, 0.1% (w/v) bovine serum albumin, and 0.5% (w/v) polyvinylpyrrolidone. Homogenates were filtered through muslin cloth, subjected to differential centrifugation, and then fractionated with a 0.6 M Suc cushion (as in Lund et al., 1998). The mitochondrial fraction was collected and washed with mitochondrial isolation buffer lacking polyvinylpyrrolidone and then resuspended in 70 mM Suc, 250 mM mannitol, and 30 mM MOPS (pH 7.2). The samples were frozen at -72°C , thawed, and then centrifuged at 75,000g for 20 min. The pellet was resuspended in the same Suc-mannitol-MOPS buffer. For assays involving Suc protection, a buffer consisting of 320 mM mannitol and 30 mM MOPS (pH 7.2) was used in place of the above buffer.

Electron Transport Assays

Electron transport from Complex I through Complex III and Complex IV or Complex II through Complex III and Complex IV was measured polarographically using a

Clark-type electrode (1-mL volume; Hansatech, Norfolk, UK). Sub-mitochondrial particles were suspended in 70 mM Suc, 250 mM mannitol, and 30 mM MOPS (pH 7.2). NADH concentration was titrated to produce maximal Complex I activity (0.25 mM NADH), and activity was assayed using 0.5 mM thenoyltrifluoroacetone (an inhibitor of Complex II) and 1 mM SHAM (an inhibitor of alternative oxidase activity). For Complex II to IV, the succinate concentration required to produce maximal Complex II activity was determined to be 20 mM, and activity was assayed using 50 μM rotenone (to inhibit Complex I) and 1 mM SHAM. ATP was added 30 s before succinate during the assay to activate the complex (Douce et al., 1987). For each assay, components were added from concentrated stocks to generate the specified concentration. For NaCl, the concentrations were 50, 75, 100, and 200 mM. Betaine and L-Pro concentrations were 2.5, 5, 10, and 15 μM . Suc concentrations were 10, 25, 50, and 100 mM. The ascorbate, glutathione, and α -tocopherol concentrations were 5, 10, 20, and 30 mM. To check for APX activity, 0.5 mM p-CMB, an inhibitor of APX (Mittler and Zilinskas, 1993), was added to ascorbate and glutathione assays (optimal concentration was determined by titration in multiple assays, data not shown). In an alternate manner, DTT was added at a concentration of 0.1 mM to ascorbate and glutathione assays as an inhibitor of APX when H₂O₂ is present (Chen and Asada, 1992). The concentrations of catalase (Calbiochem, La Jolla, CA) and Cu/Zn SOD (Calbiochem) were 50, 100, and 200 $\mu\text{g mL}^{-1}$. Purified mitochondrial sHsps concentration were 6.25, 12.5, and 25 $\mu\text{g mL}^{-1}$ (see below). Disruption of mitochondrial sHsp protection by preincubations with antimitochondrial sHsp antiserum for 5 min was titratable, and maximal disruption occurred by 50 $\mu\text{L mL}^{-1}$ of antiserum. Protectants were added after the establishment of linear electron transport rate (6–8 min) after which a linear rate was re-established (6–8 min) and then 100 mM NaCl was added.

Purification of Mitochondrial sHsp

Mitochondrial sHsps were purified from heat-stressed corn plants, using a method modified from Lee and Vierling (1998). Intact plants were heat stressed during the day by linearly increasing growth chamber temperatures over 2 h from 30°C to 42°C, and then holding plants at 42°C for 4 h, at which time plants were harvested. Mitochondria were isolated from roots and hypocotyls, as above, and resuspended in a protein purification buffer containing 20 mM Tris (pH 8.0), 10 mM Suc, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM ϵ -amino caproic acid, 1 μM leupeptin, and 1 μM antipain. Mitochondrial proteins were then fractionated by ammonium sulfate precipitation [25%, 50%, 75%, and 100% (w/v) (NH₄)₂SO₄]; mitochondrial sHsps were precipitated at 75% (determined by SDS-PAGE and immunoblotting). Following this, the sHsp-enriched fraction was resuspended in protein purification buffer and then diluted 100-fold with 20 mM Tris (pH 8.0), to ensure that residual (NH₄)₂SO₄ was less than 30 mM. The proteins in this fraction were separated by

anion-exchange chromatography, using DEAE Sepharose (CL-6B, Sigma, St. Louis) in a 13- × 2.5-cm gravity flow column. Proteins were eluted from the column using a step-gradient of NaCl in 20 mM Tris (pH 8.0; 100, 200, 300, 400, and 500 mM NaCl). Anion-exchange fractions containing sHsp were determined by SDS-PAGE and immunoblotting; purity of sHsp in the sHsp-containing fractions was estimated by silver staining and confirmed by immunoblotting. The most enriched fraction was estimated to be about 95% sHsp, and aliquots of this sHsp-enriched fraction were used in electron-transport protection assays. To confirm that any observed protection of electron transport by this fraction was attributable to sHsp, some aliquots were first mixed with polyclonal antiserum to mitochondrial sHsp to disrupt the interaction of sHsp with mitochondrial proteins and prevent sHsp protection (as in Downs et al., 1998, 1999b; Heckathorn et al., 1998); pre-immune serum was used as a negative control for antibody disruption of sHsp (see results). In addition, other anion-exchange fractions were assayed for their ability to protect electron transport and no other fraction tested provided protection (data not shown).

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