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Comparative Study of Thermal Degradation of Iron–Sulfur Proteins in Spinach Chloroplasts and Membranes of Thermophilic Cyanobacteria: Mössbauer Spectroscopy

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Abstract—Mössbauer spectra of chloroplasts isolated from spinach plants grown in a mineral medium enriched with ^{57}Fe and Mössbauer spectra of native membranes of the thermophilic cyanobacterium *Synechococcus elongatus* contain a broad asymmetric doublet typical of the iron–sulfur proteins of Photosystem (PS) I. Exposure of chloroplasts to temperatures of 20-70°C significantly modifies the central part of the spectra. This spectral change is evidence of decreased magnitude of the quadrupole splitting. However, the thermally induced doublet (ΔQ = 3.10 mm/sec and δ = 1.28 mm/sec) typical of hydrated forms of reduced (divalent) inorganic iron is not observed in spinach chloroplasts. This doublet is usually associated with degradation of active centers of ferredoxin, a surface-exposed protein of PS I. The Mössbauer spectra of photosynthetic membranes of spinach chloroplasts and cyanobacteria were compared using the probability distribution function of quadrupole shift (1/2 quadrupole splitting ΔQ) of trivalent iron. The results of calculation of these functions for the two preparations showed that upon increasing the heating temperature there was a decrease in the probability of the presence of native iron–sulfur centers F_X, F_A, and F_B (quadrupole shift range, 0.43-0.67 mm/sec) in heated preparations. This process was also accompanied by an increase in the probability of appearance of clusters of trivalent iron. This increase was found to be either gradual and continuous or abrupt and discrete in photosynthetic membranes of cyanobacteria or spinach chloroplasts, respectively. The probability of the presence of the iron–sulfur centers F_X, F_A, and F_B in chloroplasts abruptly decreases to virtually to zero within the temperature range critical for inhibition of electron transport through PS I to oxygen. In cyanobacteria, both thermal destruction of iron–sulfur centers of PS I and functional degradation of PS I are shifted toward a higher temperature. The results of this study suggest that the same mechanism of thermal destruction of the PS I core occurs in both thermophilic and mesophilic organisms: destruction of iron–sulfur centers F_X, F_A, and F_B, release of oxidized (trivalent) iron, and its accumulation in membrane-bound iron-oxo clusters.

Key words: Mössbauer spectroscopy, spinach, thermophilic cyanobacteria, photosynthesis, Photosystem I, iron–sulfur centers, thermal destruction

Mössbauer spectroscopy of biological materials is a unique noninvasive method of elucidation of correlation between microstructural changes of the active sites of proteins and inhibition of their functional activity. This is particularly true in the case of proteins containing iron in the active site. Even slight changes of such parameters of Mössbauer spectra as isomer shift or quadrupole splitting induced by external factors (temperature, ionizing radiation, etc.) can be regarded as evidence of initial stages in reorganization and/or structural degradation of a protein. These spectral parameters provide information about changes in the electronic structure of iron. In some cases, Mössbauer spectral changes indicate the release of iron from the active sites during protein degradation.

In the preceding works [1, 2], we studied the Mössbauer spectra of the thermophilic cyanobacterium *Synechococcus elongatus* and showed that thermal destruction of functional activity is closely associated with structural degradation of iron–sulfur proteins. It was also found that thermal degradation of ferredoxin differs

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significantly from thermal degradation of other iron–sulfur proteins of PS I (F_A, F_B, and F_X). These proteins are bound to the photosynthetic membrane [2] and have higher thermal resistance than ferredoxin. At temperatures above 60°C, there was an abrupt increase in the rate of destruction of iron–sulfur centers of ferredoxin and accumulation of reduced iron. At 80°C, the active site of ferredoxin is completely destroyed and the content of Fe^{2+} reaches its maximum level. In contrast to ferredoxin, the degree of thermal destruction of the iron–sulfur centers F_A, F_B, and F_X at 80°C did not exceed 50%. There was a quantitative correlation between the decrease in the amplitude of the Mössbauer spectra of centers F_A, F_B, and F_X and appearance of spectral components of oxidized iron. This correlation can be explained by the thermally induced increase in the concentration of clusters of oxidized iron caused by degradation of iron–sulfur centers.

The primary structure of iron–sulfur proteins of PS I of thermophilic bacteria (ferredoxin, in particular) is very similar to the primary structure of analogous proteins of mesophilic organisms [3]. High thermal stability of *S. elongatus* ferredoxin was attributed to the specific spatial structure of this protein (large hydrophobic core formed by additional salt bridges) [3, 4]. However, this structural rearrangement does not involve the amino acid surrounding of the iron–sulfur centers of ferredoxins in thermophilic and mesophilic organisms. Therefore, it can be suggested that mechanisms of thermal inactivation of iron–sulfur centers of PS I in thermophilic and mesophilic organisms are similar.

The goal of this work was to compare the thermally induced changes in the state of iron in iron–sulfur centers of spinach chloroplasts and photosynthetic membranes of the thermophilic cyanobacterium *Synechococcus elongatus* using Mössbauer spectroscopy over the temperature range of functional degeneration of photosynthetic activity.

MATERIALS AND METHODS

Spinach plants were grown hydroponically in a mineral medium as described in [5]. Plants for Mössbauer spectroscopic experiments were grown in medium enriched with ^{57}Fe. Chloroplasts were isolated as described in [6], suspended in a buffer solution containing 50 mM Tricine, 10 mM NaCl, and 400 mM sucrose (pH 7.8), and stored in liquid nitrogen until use.

The cyanobacterium *S. elongatus* was grown and its photosynthetic membranes were isolated as described earlier [7, 8].

Mössbauer spectra of chloroplasts were measured at 80K using an electrodynamic spectrometer with uniformly accelerated motion of the sample (6-10 mg chlorophyll per 0.6 ml sample) relative to a radiation source (^{57}Co in a Rh matrix). Mössbauer spectra were simulated using the computer programs Univem (MOSTEK, Rostov) and MSTools (Moscow State University).

Experiments were carried out as follows. The temperature dependences of the Mössbauer spectra were measured using the same sample of spinach chloroplasts after sequential heating for 5 min intervals at different temperatures. For this procedure, the initial samples were exposed to a given temperature for 5 min in a thermostat and then frozen in liquid nitrogen. After the Mössbauer spectrum had been measured, the sample was slowly thawed (40-45 min), exposed to a higher temperature, and its Mössbauer spectrum was measured again.

The rate of the electron transfer mediated by PS I was measured by the rate of oxygen uptake at 20°C in the presence of 4 μM Diuron, 40 μM 2,6-dichlorophenol indophenol, 1 mM sodium ascorbate, and 0.1 mM methyl viologen. To measure the thermal stability of the oxygen uptake reaction rate, membrane samples were exposed to a given temperature for 5 min in a thermostat and then cooled to room temperature. A new sample was taken for measurements at each temperature. Oxygen uptake rate was measured using a Clark electrode. A typical rate of oxygen uptake by chloroplasts in the presence of uncoupler at room temperature was 50 μmol O_2 per h per mg chlorophyll.

Tricine was from Sigma (USA). The other reagents were chemical purity grade (or higher purity grade) products of domestic manufacturers.

RESULTS AND DISCUSSION

Figure 1 shows that although the thermal stability of functional activity of PS I in spinach chloroplasts is rather high (Fig. 1a), it is significantly lower than in the membranes of the cyanobacterium *S. elongatus* (Fig. 1b). A 50% inhibition of the electron transport through spinach PS I to oxygen was observed only at 62°C, whereas the thermotropic structural transition of the PS I protein complex in general is observed at 57°C [9].

Mössbauer spectra of spinach chloroplasts are shown in Fig. 2. Like the spectrum of the thermophilic cyanobacterium *S. elongatus* described in [2, 10], these spectra contain a broad asymmetric doublet typical of iron-containing PS I centers [11-13]. Sequential exposure of spinach chloroplasts to 35, 40, 65, and 70°C modified the central part of the spectrum. As in case of the thermophilic cyanobacterium *S. elongatus* [1, 2], heating decreased the spectral resolution. This suggests the formation of spectral components with lower quadrupole splitting typical of cluster forms of oxidized iron.

However, the Mössbauer spectra of spinach chloroplasts heated to 70°C do not contain the thermally induced doublet with quadrupole splitting $\Delta Q = 3.10$ mm/sec and isomeric shift $\delta = 1.28$ mm/sec. A similar doublet

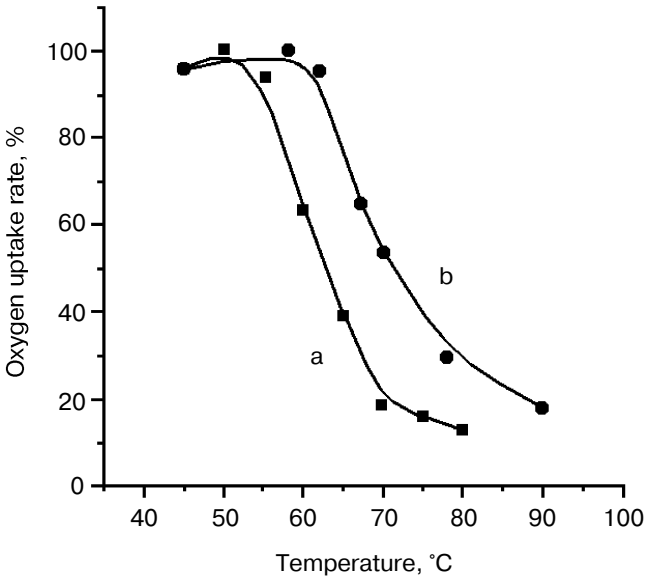

Fig. 1. Thermal stability of electron transport through PS I in spinach chloroplasts (a) and photosynthetic membranes of the cyanobacterium *S. elongatus* (b). Dichlorophenol indophenol and methyl viologen were used as electron donor and acceptor, respectively. The electron transfer rates were measured in the presence of Diuron.

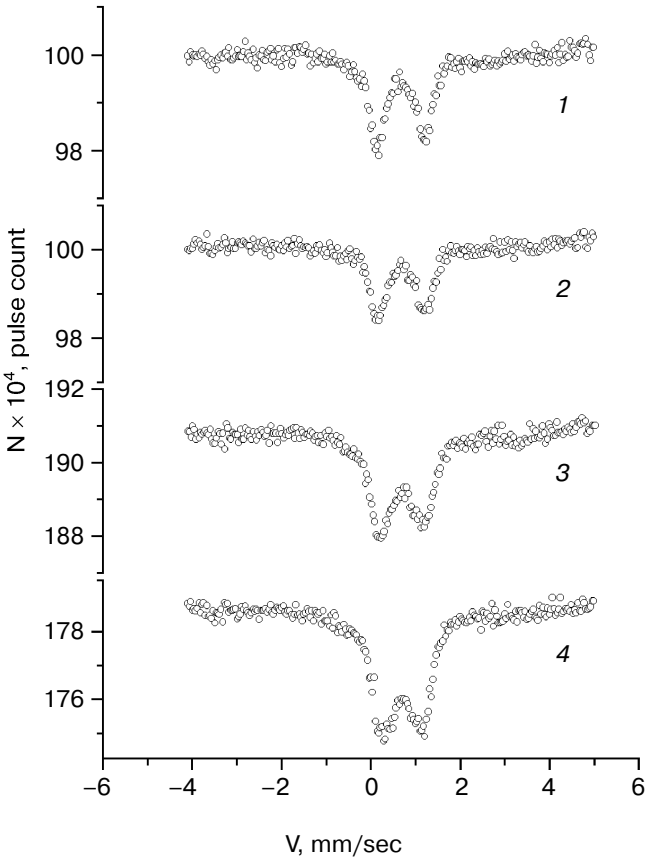

Fig. 2. Mössbauer spectra (80K) of spinach chloroplasts exposed for 5 min to 35 (*1*), 40 (*2*), 65 (*3*), and 70°C (*4*). The chloroplasts were suspended in buffer solution containing 25% glycerol.

was observed in the preceding study of thermal degradation of photosynthetic membranes of thermophilic cyanobacteria. It was suggested that this doublet is a spectral indicator of degradation of active centers of ferredoxin, a surface protein of PS I. It was shown in [3] that a large number of salt bridges in ferredoxins from thermophilic organisms make the molecular structure of

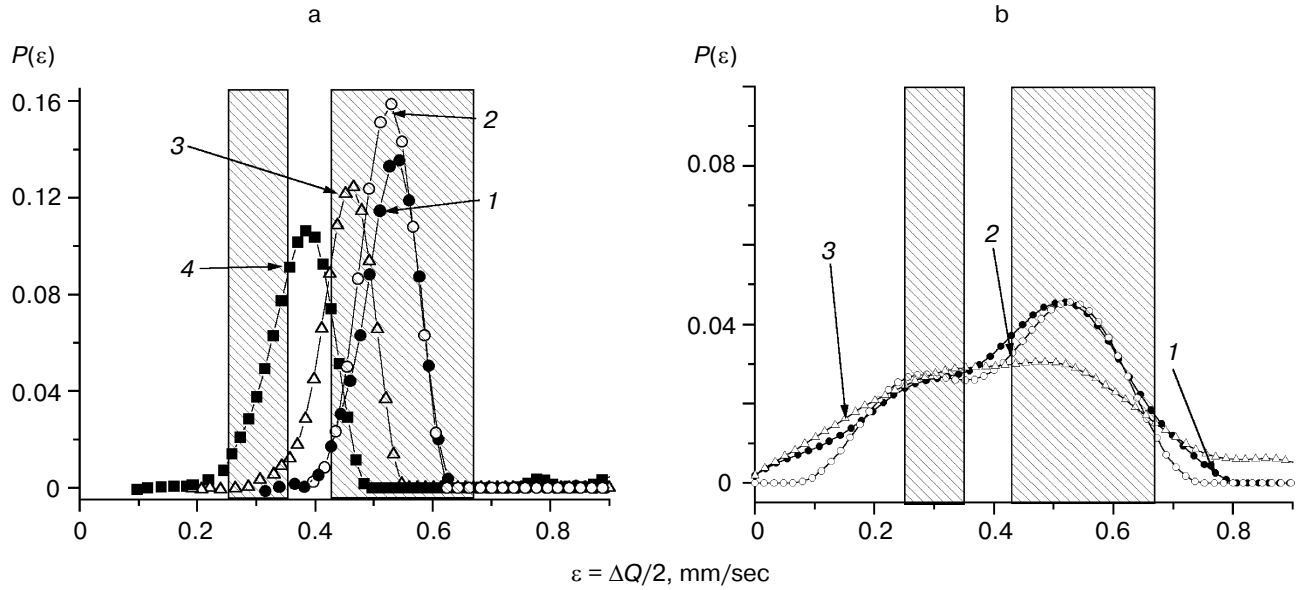

Fig. 3. Distribution function of quadrupole shift P(ε) in Mössbauer spectra of spinach chloroplasts (a) and photosynthetic membranes of the cyanobacterium *S. elongatus* (b) exposed to different temperatures: 35 (*1*), 40 (*2*), 65 (*3*), and 70°C (*4*) (a); 50 (*1*), 60 (*2*), and 70°C (*3*) (b).

these proteins more compact. It is conceivable that compact spatial structure of ferredoxins of thermophilic organisms provides for effective reduction of the active iron center during its partial thermal denaturation mediated by sulfur-containing molecular fragments [14].

The following approach to comparative analysis of Mössbauer spectra of spinach chloroplasts and thermophilic cyanobacteria was suggested. An iterative variant of the general method of regularization described in [15] was used to restore the quadrupole distribution probability functions (in the case considered above, 1/2 quadrupole splitting ΔQ) for trivalent iron in all spectra studied in this work.

Such functions calculated for the Mössbauer spectra of spinach chloroplasts and photosynthetic membranes of thermophilic cyanobacteria heated to different temperatures are shown in Figs. 3a and 3b, respectively. The quadrupole distribution probability functions of cyanobacterial membranes were restored after the spectral component of divalent iron had been subtracted. The divalent iron appeared in membranes due to degradation of ferredoxin. Two areas of the quadrupole shift of 0.25-0.35 and 0.43-0.67 mm/sec typical of oxidized iron clusters [16] and oxidized forms of the iron–sulfur proteins of PS I (F_A, F_B, F_X, and ferredoxin) [1, 11-13], respectively, are shaded in Fig. 3. As the temperature of heating of spinach chloroplasts increased, there was a decrease in the probability of observation of native iron–sulfur proteins and a corresponding increase in the probability of observation of oxidized iron clusters. Similar effects were induced by heating thermophilic cyanobacterial membranes. However, the probability changes in photosynthetic membranes of cyanobacteria or spinach chloroplasts were either gradual and continuous or abrupt and discrete, respectively. Therefore, the iron–sulfur centers F_A, F_B, and F_X of cyanobacteria are thought to undergo gradual thermal degradation. It should also be noted that the width of the probability distribution function of quadrupole shift in spectra of cyanobacteria is significantly larger than in spectra of spinach chloroplasts. This was due to a larger spread of the quadrupole splitting values in the iron–sulfur centers F_A, F_B, and F_X of cyanobacteria.

Thus, the results of this study suggest that the same mechanism of thermal destruction of the PS I core is valid in both thermophilic and mesophilic organisms. This mechanism includes destruction of iron–sulfur centers F_X, F_A, and F_B, accompanied by formation of a large fraction of membrane-bound multiatomic iron clusters. It is conceivable that different temperature dependence of these processes in cyanobacteria and spinach chloroplasts is due to the different thermal resistance of these organisms. Photodestruction of PS I caused by cold shock and exposure of membranes of mesophilic photosynthesizing organisms to some other extreme factors is also accompanied by destruction of iron–sulfur centers F_X, F_A, and F_B [17, 18]. Perhaps, release of oxidized iron from the iron–sulfur center and its accumulation in membrane-bound iron-oxo clusters constitute the central element of the process of destruction of the PS I core in various groups of organisms with different thermal resistance.

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