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Bicarbonate Stabilizes Isolated D1/D2/Cytochrome b_{559} Complex of Photosystem 2 against Thermoinactivation

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Abstract—It has been shown that thermoinactivation of the isolated D1/D2/cytochrome b_{559} complex (RC) of photosystem 2 (PS-2) from pea under anaerobic conditions at 35°C in 20 mM Tris-HCl buffer (pH 7.2) depleted of HCO_3^-, with 35 mM NaCl and 0.05% *n*-dodecyl-*β*-maltoside, results in a decrease in photochemical activity measured by photoreduction of the PS-2 primary electron acceptor, pheophytin (by 50% after 3 min of heating), which is accompanied by aggregation of the D1 and D2 proteins. Bicarbonate, formate, and acetate anions added to the sample under these conditions differently influence the maintenance of photochemical activity: a 50% loss of photochemical activity occurs in 11.5 min of heating in the presence of bicarbonate and in 4 and 4.6 min in the presence of formate and acetate, respectively. The addition of bicarbonate completely prevents aggregation of the D1 and D2 proteins as opposed to formate and acetate (their presence has no effect on the aggregation during thermoinactivation). Since the isolated RCs have neither inorganic Mn/Ca-containing core of the water-oxidizing complex nor nonheme Fe^{2+}, it is supposed that bicarbonate specifically interacts with the hydrophilic domains of the D1 and D2 proteins, which prevents their structural modification that is a signal for aggregation of these proteins and the loss of photochemical activity.

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Key words: photosystem 2, isolated D1/D2/cytochrome b_{559} complex, bicarbonate, thermoinactivation

Bicarbonate ion (HCO_3^-) is known to have a unique effect on the reactions in photosystem 2 (PS-2) [1, 2]. The idea of possible involvement of bicarbonate in water photooxidation was first proposed by Warburg as early as the 1960s [3]; indisputable evidence of the involvement of HCO_3^- in the function of PS-2 was obtained later by Govindjee [4] and Stemler [5]. Data obtained by different research teams during the past 15 years show the requirement of bicarbonate both on the "acceptor" side of PS-2, where it binds to the nonheme iron cation (Fe^{2+}) located between the primary (Q_A) and secondary (Q_B) quinone electron acceptors [1, 3, 4, 6] and on the "donor" side of PS-2 [7-12]. A series of works convincingly demonstrated the necessity of HCO_3^- for the photosynthetic oxidation of water [7-12]. Bicarbonate was shown to stimulate electron donation from exogenous Mn^{2+} added to manganese-free preparations of PS-2 [7-9]. Based on electrochemical data, it was shown that this unique ability of bicarbonate could be associated with formation of Mn-bicarbonate complex providing a substantial decrease in the redox potential of Mn^{II} (to 0.52 V) [10]. HCO_3^- was shown to be a cofactor for the assembly of the inorganic core of the water-oxidizing complex (WOC), $Mn_4O_xCaCl_y$, during photoactivation [7, 9, 11, 12]. Some data give evidence of probable participation of bicarbonate in proton acceptance during photosynthetic water oxidation [13]. The results suggest that bicarbonate is an integral component of the WOC necessary for its function and stabilization [7-12]. However, in spite of numerous investigations showing the participation of bicarbonate in PS-2 function, its binding site and the mechanism of stabilizing effect are still unknown. Most of these data suggest that the action of bicarbonate in PS-2 function is determined by its interaction with nonheme iron (on the acceptor side of PS-2) [1, 3, 4, 6] or with the Mn cluster of the WOC inorganic core (on the donor side of PS-2) [7-10]. However, it is not improbable that HCO_3^- can interact not only with the metal components of PS-2, but also with the amino acid residues of its protein components. This possibility was confirmed by our two recent

Abbreviations: DM, *n*-dodecyl-*β*-maltoside; PS-2, photosystem 2; RC, reaction center; TMPD, *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine dihydrochloride; WOC, water-oxidizing complex.

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works giving evidence of the protective effect of bicarbonate on the maintenance of structural organization of PS-2 pigment–protein complex [14] and on the structure of its separate component: a manganese-stabilizing protein (PsbO protein) that is a part of the WOC [15]. Previously, it was shown that bicarbonate maintains the binding of three hydrophilic proteins (PsbO, PsbP, and PsbQ) during the treatment of PS-2 membrane fragments with urea and at acidic pH values [14]. The addition of HCO_3^- ion to PsbO protein solution at acid pH values causing conformational changes in the protein globule and enhancing the accessibility of its hydrophobic regions to the water phase returns its structure into a native compact state typical of the protein at neutral pH values [15]. The effect of bicarbonate is specific: structurally similar formate and acetate ions did not show these properties. In these works, it was suggested that the specific effect of bicarbonate is determined by its interaction with separate positively charged amino acid residues of the PsbO protein. Comparison of the protective effects of glycine betaine and HCO_3^- ion during photo- and thermoinactivation of thylakoids and membrane fragments of spinach PS-2 leads to a conclusion that both of them stabilize the pigment–protein complex of PS-2, but the protective nature of these components is different [16]. Glycine betaine as a nonspecific zwitterionic osmolyte at high concentrations stabilizes the macrostructure of PS-2 and provides the maintenance of native configuration of its protein components. The protective effect of bicarbonate is exhibited at concentrations lower by three orders of magnitude compared to glycine betaine, demonstrating its specific interaction with PS-2 components. The effect of bicarbonate is probably mediated by its interaction not only with the metal components of PS-2 (nonheme iron, Fe^{2+}, and Mn cation) but also with the proteins carrying the main RC and WOC cofactors of photosystem 2. Hence, a convenient model for the study of bicarbonate effect on the structural organization and function of PS-2 pigment–protein complex is the isolated D1/D2/cytochrome b_{559} (RC) complex containing neither quinones Q_A and Q_B, nor Mn cluster, nor nonheme iron. They contain D1 and D2 proteins, the α- and β-subunits of cytochrome b_{559}, and the *PsbI* gene product, which bind four molecules of chlorophyll *a*, two molecules of pheophytin *a*, and 1-2 molecules of β-carotene [17]. This RC is the minimal pigment–protein complex of PS-2 capable of photochemical charge separation. Dark thermoinactivation has been used in the present work for reducing the functional activity of the D1/D2/cytochrome b_{559} complex and for damaging its structural organization. It is known that the heating of thylakoids, subchloroplast membrane fragments, and isolated "core" oxygen-evolving complex of PS-2 in the dark results not only in disturbance of their functional activity (measured by photoinduced changes in the yield of PS-2 chlorophyll fluorescence and oxygen evolution rate [18-23]) but also in substantial structural changes within the complex: the release of three hydrophilic proteins (PsbO, PsbP, and PsbQ) and Mn from the binding sites in the WOC [18-23], decrease in the content of light-harvesting complex (LHC), and decrease in the size of PS-2 particles [21].

The objective of this work was to investigate the potential stabilizing effect of bicarbonate on the functional activity and structural organization of D1/D2/cytochrome b_{559} complex isolated from pea leaves during dark thermoinactivation.

MATERIALS AND METHODS

The following agents were used in the work: Tris, Triton X-100, *n*-dodecyl-*β*-maltoside (DM), *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), acrylamide, SDS (Sigma, USA); other reagents were produced by AppliChem.

The D1/D2/cytochrome b_{559} complex was obtained from pea (*Pisum sativum*) leaves as described [24]. RC preparations contained the D1 (32 kDa) and D2 (34 kDa) proteins and cytochrome b_{559} (9 kDa). Two pheophytin *a* molecules corresponded to 4-5 chlorophyll *a* molecules.

Concentrated RC preparations before use were diluted 10-20 times (to a final concentration of 2 μg chlorophyll/ml) in 20 mM Tris-HCl buffer, pH 7.2, containing 35 mM NaCl, and 0.05% DM. For reducing the content of dissolved HCO_3^-/CO_2, the buffer prepared immediately before the measurement using dry Tris and fresh HCl solution was purged with air that was first passed through a 50% NaOH solution and a 20-cm Ascarite layer. Hereinafter, it will be termed as a "HCO_3^--depleted" buffer.

Thermoinactivation was performed in a CH-100 thermostat (BIOSAN).

The photochemical activity of D1/D2/cytochrome b_{559} complex was measured with an Agilent 8453 spectrophotometer (USA) by the value of reversible photoinduced changes in absorption (ΔA) at 450 nm (in one of the maximums of the ΔA spectrum associated with photoreduction of the PS-2 primary electron acceptor, pheophytin, to $Pheo^-$) in the presence of 150 μM exogenous electron donor TMPD and 10 mM sodium ascorbate as described [25]. Thermoinactivation and measurements were performed under anaerobic conditions created with the aid of a system containing glucose (20 mM), glucose oxidase (0.1 mg/ml), and catalase (0.039 mg/ml) [26]. A sample was put into a cuvette (1 cm) and illuminated with actinic light (100 Wt/m^2) through a glass filter (KS-11) transmitting light of $\lambda > 600$ nm via a light guide.

Thermally induced spectral changes were revealed by measuring the absorption spectra of preparations with a SF-102 spectrophotometer (Russia).

The protein analysis of tested preparations was carried out by 12-18% gradient polyacrylamide gel elec-

trophoresis in the presence of 0.1% SDS [27]. The upper electrode Tris-glycine buffer (150 mM, pH 8.3) contained 0.1% SDS. Proteins in the gel were stained by Coomassie G-250. After staining, the gels were scanned. The relative quantity of proteins and protein aggregates was analyzed with Total Lab v.2.01 software. The relative quantity of each protein in the respective band of the electrophoregram was taken as 100%.

RESULTS

As shown previously, the isolated D1/D2/cytochrome b_{559} complex of PS-2 reaction center do not contain quinone acceptors Q_A and Q_B [17]. As a result, the primary photochemical reaction in this complex is limited by electron transfer from the excited primary electron donor P680* to one of the pheophytin *a* (Pheo *a*) molecules [28]. The formed primary ion-radical pair [$P680^+$ $Pheo^-$] has a very short lifetime (~10^{-8} sec) and is quickly recombined into the initial state [P680 Pheo] or triplet state [29]. In the presence of an exogenous electron donor that is able to effectively donate electrons to $P680^+$ by competing with charge recombination, the long-living state [P680 $Pheo^-$] accumulates under illumination of RC complex in the absence of oxygen or other electron acceptors [30]. In this case, the amplitude of ΔA associated with reversible photoreduction of Pheo to $Pheo^-$ can be used as a measure of RC photochemical activity. Reduced TMPD was used as an exogenous electron donor [25]. Tris-HCl buffer, pH 7.2, was used as the buffer; as shown previously [31], stability of the D1/D2/cytochrome b_{559} complex in the dark (and under photoinactivation) depends on the pH of the medium, reaching its maximum at neutral pH values. The presence of salt ions in the sample can influence the stability of pigment–protein complex via electrostatic interactions. The measurements were made against 35 mM NaCl to eliminate the influence of ionic strength on addition of sodium bicarbonate.

Figure 1 shows that the amplitude of reversible photoinduced ΔA at 450 nm (ΔA_{450}) in the D1/D2/cytochrome b_{559} complex of PS-2 heated at 35°C for 3 min in the absence of other additives decreases by 54% (*2*) compared to the control (*1*). However, the ΔA_{450} signal decreased less (by only 28% compared to the control) if 2 mM of bicarbonate was added to the sample before the treatment (*3*). Addition of the same amounts of formate (*4*) and acetate (*5*) that are structurally similar to HCO_3^- before thermal treatment reduced the effect of thermoinactivation but to a lesser degree than in the case of bicarbonate (the signal decreased by 42 and 39%, respectively, compared to the control). The ΔA_{450} signal decreased after simultaneous addition of bicarbonate and formate to the sample before heating (*6*) to the same extent as with bicarbonate only.

It should be mentioned that analogous results were obtained from measuring the amplitude of reversible photoinduced ΔA at 682 nm.

Figure 2 shows the dependence of reversible change in absorption at 450 nm on the time of incubation of the D1/D2/cytochrome b_{559} complex at 35°C. One can see that photochemical activity of the complex at a longer time of heating at 35°C in the HCO_3^--depleted medium in the absence of other additives drops to 50% of the initial level after 3 min of incubation (curve *1*). When thermal inactivation was performed in the presence of 2 mM bicarbonate, the curves of the drop in photochemical activity in the experiment (with bicarbonate, curve *2*) and the control (curve *1*) demonstrate substantial difference from the very first minutes of heating. The difference between them increases along with the time of heating, so that the 50% loss of activity in the presence of bicarbonate is observed only after 11.5 min of incubation (curve *2*). When RC complex was thermally inactivated in the presence of 2 mM formate or 2 mM acetate, there was no significant difference between the curves of the drop in photochemical activity in the experiment (curves *3* and *4*) and the control (curve *1*) and the drop in activity reached 50% at a 4- and 4.6-min incubation, respectively.

Figure 3 shows the dependence of photochemical activity of the D1/D2/cytochrome b_{559} complex during 3-min thermoinactivation under anaerobic conditions at 35°C on bicarbonate concentration. At higher concentra-

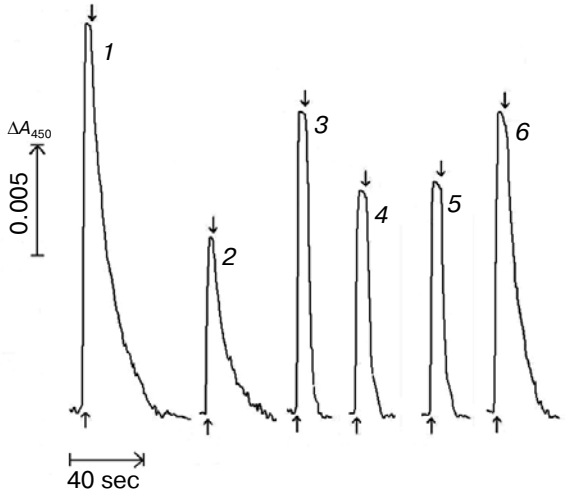

Fig. 1. Kinetics of photoinduced changes in absorption at 450 nm associated with photoaccumulation of reduced primary electron acceptor pheophytin in the isolated D1/D2/cytochrome b_{559} complex of PS-2 in the presence of reduced TMPD under anaerobic conditions before (*1*) and after 3-min heating at 35°C in HCO_3^--depleted 20 mM Tris-HCl buffer, pH 7.2, containing 35 mM NaCl and 0.05% DM (*2*) and in the same buffer after the addition of 2 mM bicarbonate (*3*), 2 mM formate (4), 2 mM acetate (*5*), or 2 mM formate and 2 mM bicarbonate simultaneously (*6*). Chlorophyll concentration in each sample was 2 μg/ml. The arrows show light switching on (↑) and off (↓).

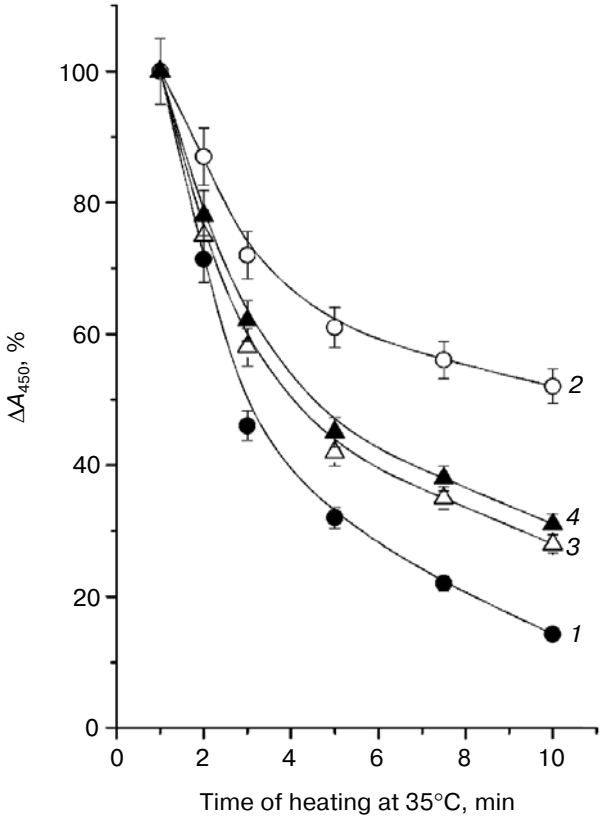

Fig. 2. Dynamics of decrease in photochemical activity of D1/D2/cytochrome b_{559} complex of PS-2 RC measured by the reversible photoinduced changes in absorption at 450 nm (ΔA_{450}) associated with photoreduction of the primary electron acceptor pheophytin in the presence of reduced TMPD during thermoinactivation under anaerobic conditions at 35°C in HCO_3^--depleted 20 mM Tris-HCl buffer, pH 7.2, containing 35 mM NaCl and 0.05% DM before (*1*) and after the addition of 2 mM bicarbonate (*2*), 2 mM formate (*3*), and 2 mM acetate (*4*).

tions its stabilizing effect increases and reaches a maximum at 2-3 mM. At higher concentrations the dependence curve is essentially unchanged. It has been shown that this amount of added bicarbonate does not change pH in the sample significantly (the pH shift is less than 0.03 unit).

In Fig. 4 (a-c), the absorption spectra of RC preparations are compared over the course of heating at 35°C under anaerobic conditions in HCO_3^--depleted medium (Fig. 4a) in the absence of other additives and in the same medium in the presence of 2 mM bicarbonate (Fig. 4b) or 2 mM formate (Fig. 4c). In the first three minutes of thermoinactivation, the character of the spectrum is unchanged in the sample without additives (Fig. 4a) and in the sample with formate, but a considerable increase in light scattering is observed as an apparent increase in optical density of the sample equivalent to 0.15 and 0.1 unit, respectively. Thermoinactivation of the complex in the presence of acetate resulted in the same increase in light scattering as with formate (data not shown). With thermoinactivation in the presence of bicarbonate, light scattering increased insignificantly (the apparent increase in optical density was equivalent to 0.005 unit) (Fig. 4b).

Figure 5a compares the results of electrophoretic analysis of proteins of the D1/D2/cytochrome b_{559} complex before (*1*) and after (*2*) 3-min thermoinactivation at 35°C under anaerobic conditions. In Fig. 5 it is clearly seen that the intensities of the bands corresponding to proteins D1 and D2 substantially decrease (by 47 and 51%, respectively) as a result of thermal treatment in the HCO_3^--depleted medium in the absence of other additives (*2*) compared to the control sample (*1*). At the same time, the broad band appearing at the running gel interface indicates the formation of aggregates. Heating of the sample in the same medium but in the presence of 2 mM bicarbonate completely prevents the aggregation (*3*), because the bands corresponding to the D1 and D2 proteins remain as

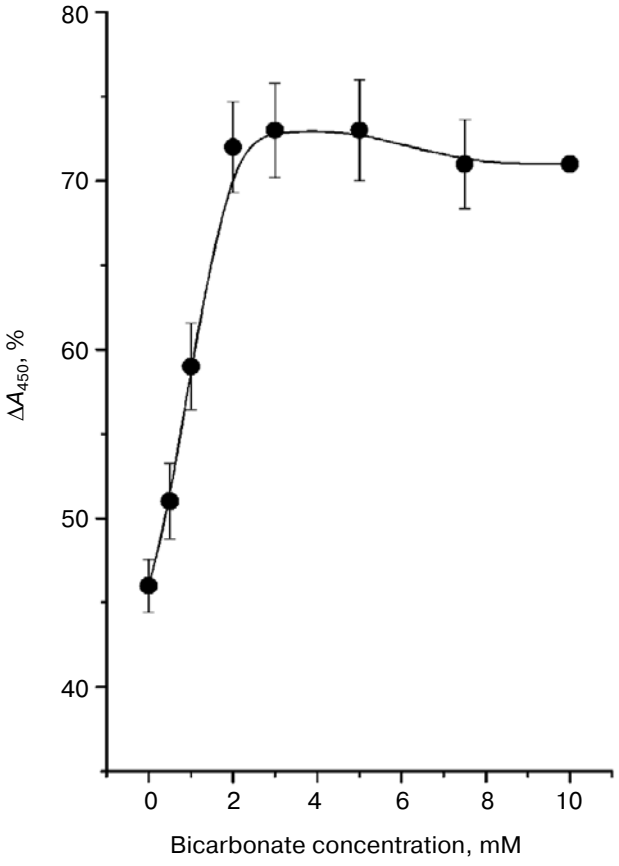

Fig. 3. Dependence of photochemical activity of the D1/D2/cytochrome b_{559} complex of PS-2 RC measured by the amplitude of reversible photoinduced changes in absorption at 450 nm (ΔA_{450}) associated with photoreduction of the primary electron acceptor pheophytin in the presence of reduced TMPD on bicarbonate concentration during 3-min thermoinactivation under anaerobic conditions at 35°C in HCO_3^--depleted 20 mM Tris-HCl buffer, pH 7.2, containing 35 mM NaCl and 0.05% DM. The initial activity corresponds to 100%.

intensive as in the control, with no changes at the gel interface. As a result of 3-min heating of RC preparations in the presence of 2 mM formate (*4*) (like during thermoinactivation in the HCO_3^--depleted medium), intensities of the bands corresponding to the D1 and D2 proteins decrease (by 30 and 52%, respectively) and a broad band at the running gel interface appears. Figure 5 also shows the dynamics of formation of D1 and D2 aggregates (Fig. 5b) and decrease in their relative content in the respective bands on the electrophoregram (Fig. 5c) under heating for 10 min at 35°C. The figure shows that the decrease in intensity of the D1 and D2 bands in the HCO_3^--depleted medium in the absence of other additives and in the presence of formate is accompanied by formation of aggregates that are clearly noticeable at the running gel interface. Neither decrease in intensity of the bands corresponding of the D1 and D2 proteins nor formation of a band at the running gel interface are observed in the presence of bicarbonate. Since the band corresponding to the α-subunit of cytochrome b_{559} (9 kDa) does not change after thermal treatment in all of the above cases, the aggregate formation seems to involve only the D1 and D2 proteins.

DISCUSSION

The results demonstrate that the isolated D1/D2/cytochrome b_{559} complex is highly sensitive to thermoinactivation. Heating for 3 min at 35°C in the HCO_3^--depleted medium results in the loss of photochemical activity by 54% compared to the control. It is accompanied by transition of almost half of the D1 and D2 proteins into aggregated forms that are clearly visible at the running gel interface during electrophoretic analysis (Fig. 5a). The presence of HCO_3^- ions in the medium during the thermoinactivation has a substantial stabilizing effect both on the photochemical activity of the complex, reducing their sensitivity to thermoinactivation 3.6-fold (Figs. 1 and 2), and on the structural organization of RC complex (Fig. 5), completely preventing the transition of the D1 and D2 proteins into aggregated forms. The effect of bicarbonate is specific, because structurally similar formate and acetate anions, though causing a small decrease in the loss of photochemical activity of the complex (by 25-27%), do not prevent the appearance of aggregated D1 and D2 protein forms under heating. The effect of bicarbonate as a salt can be excluded because all experiments were performed in the buffer with 35 mM NaCl.

PS-2 is known to be one of the most vulnerable parts of a plant under thermoinactivation [21, 32-34]. Heating has a damaging effect not only at extremely high temperatures, but also at the temperatures in the range of physiologically permissible values. It has been shown, for example, that the oxygen evolution rate in spinach thylakoids decreases under heating at a temperature of only 30-40°C [23, 35]. Most of the studies of PS-2 thermoin-

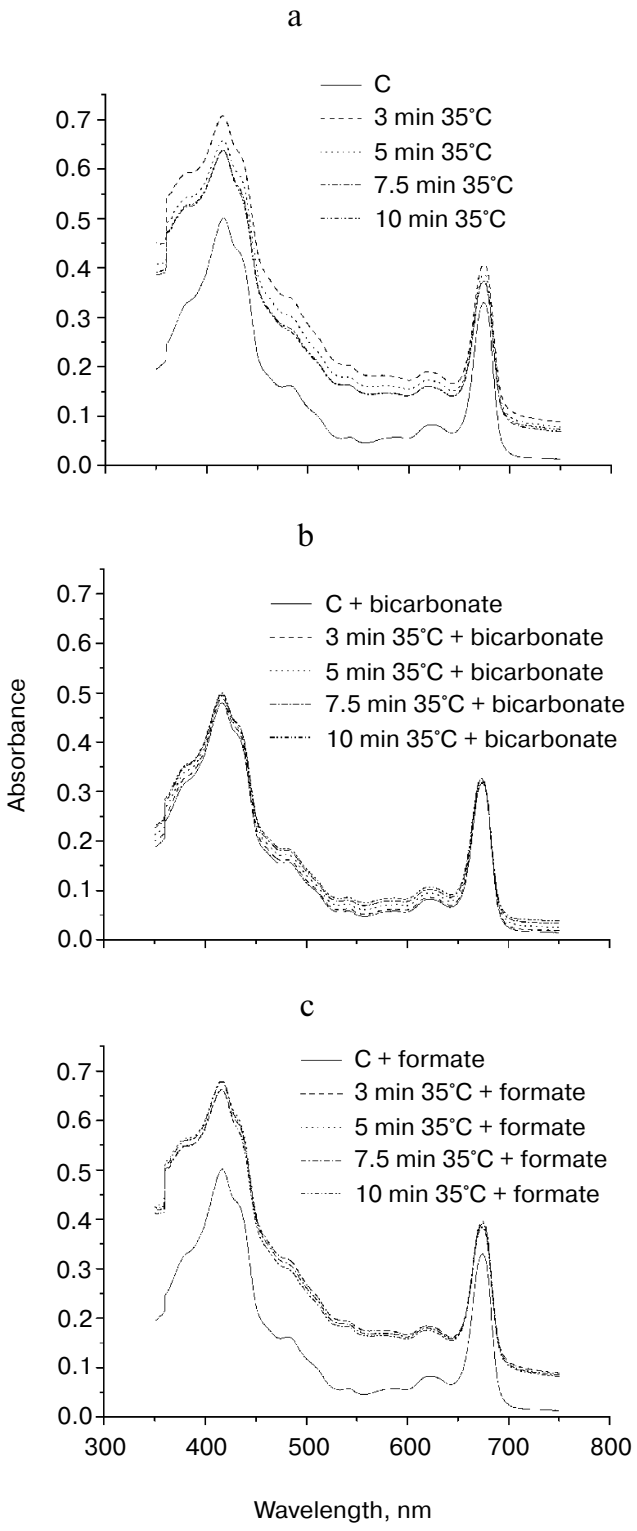

Fig. 4. Comparison of absorption spectra of the D1/D1/cytochrome b_{559} complex before (C, solid line) and after heating at 35°C under anaerobic conditions for 10 min in the HCO_3^--depleted 20 mM Tris-HCl buffer (pH 7.2) containing 35 mM NaCl and 0.05% DM (a) and in the same buffer after the addition of 2 mM bicarbonate (b) or 2 mM formate (c).

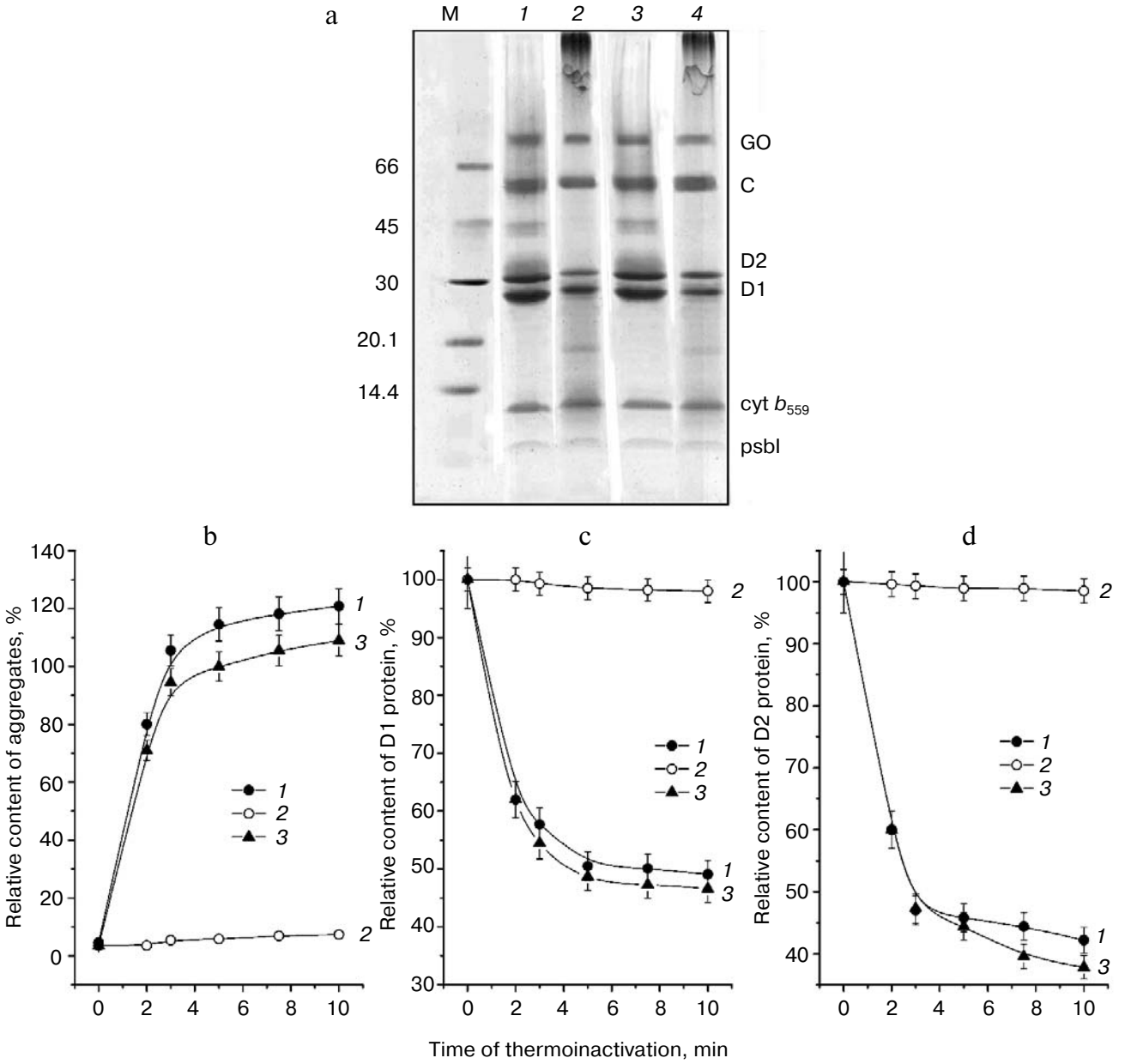

Fig. 5. Study of protein composition of the D1/D2/cytochrome b_{559} complex of PS-2 during thermoinactivation under anaerobic conditions at 35°C in HCO_3^--depleted 20 mM Tris-HCl buffer (pH 7.2) containing 35 mM NaCl and 0.05% DM. a) Electrophoregram of the complex before (*1*) and after heating without additives (*2*) and in the presence of 2 mM bicarbonate (*3*) or 2 mM formate (*4*); b-d) dynamics of formation of the aggregates of protein components of the D1/D2/cytochrome b_{559} complex (b) and the dynamics of decrease in the relative content of D1 (c) and D2 (d) proteins in the respective bands on the electrophoregram under thermoinactivation without additives (*1*), in the presence of 2 mM bicarbonate (*2*), and in the presence of 2 mM formate (*3*); M, markers containing albumin (66 kDa), ovalbumin (45 kDa), carboanhydrase (30 kDa), trypsin (20.1 kDa), and α-lactalbumin (14.4 kDa). GO, glucose oxidase; C, catalase.

activation were performed in chloroplasts, subchloroplast fragments enriched in PS-2, and isolated "core" oxygen-evolving complex of PS-2. Research into photochemical activity of such preparations under heating has shown that disturbances are observed both on the "acceptor" [36-38] and "donor" sides of PS-2 [21, 34]. The binding site of the secondary quinone acceptor Q_B is disturbed on the "acceptor" side under heat shock [39, 40].

The decrease in oxygen evolution rate observed on the donor side of PS-2 under thermoinactivation [34] is accompanied by structural changes in the PS-2 complex [18-23].

Publications devoted to the study of thermoinactivation of isolated D1/D2/cytochrome b_{559} complex are limited. One of them presents the study of temperature dependence of the secondary structure of isolated RC

[41]. FTIR spectroscopy shows that fundamental changes in the IR spectrum occur in the temperature range of 40-45°C, being evidence of high sensitivity of the secondary RC structure to thermal treatment. Appearance of the 1686- and 1622-cm^{-1} bands in the IR spectrum on temperature increase is evidence of RC protein aggregation as an irreversible process caused by the thermal treatment. The heating-induced aggregation of RC protein was also observed in this work. At present, numerous studies confirm that the aggregation of D1 protein with neighboring components (D2, CP43, and cytochrome b_{559}) is a general phenomenon observed both *in vitro* and *in vivo* under photo- and thermoinactivation of PS-2 [40]. One of the supposed regions of D1 protein through which it is bound to the nearby RC proteins is the E226-E244 region of the DE-loop located in immediate proximity to the plastoquinone Q_B-binding site [42, 43]. The stabilizing effect of betaine during thermoinactivation of isolated RC has been studied previously [44]. It was shown that the addition of the zwitterionic osmolyte betaine (which is supposed to interact with amino groups of the peptide bond of proteins [45] and thereby to stabilize the conformation of protein platform) considerably reduces the sensitivity of isolated RC to thermoinactivation by raising the semi-inactivation temperature from 29 to 35°C. The authors of that work noted that just stabilization of the protein framework of the D1/D2/cytochrome b_{559} complex is necessary for their stability to elevated temperatures. We observed a similar effect using not the zwitterionic osmolyte but the HCO_3^- ion for stabilization of the complex structure. It is important to note that the stabilizing effect of bicarbonate manifests itself at a concentration 1000 times lower than in the case of glycine betaine, demonstrating the high specificity of the bicarbonate effect.

Since the D1/D2/cytochrome b_{559} complex contains neither WOC inorganic core nor nonheme iron, this suggests that the stabilizing effect of bicarbonate is a consequence of its interaction with amino acid residues of protein components. Both D1 and D2 proteins (carrying the major cofactors of PS-2 RC) contain five transmembrane α-helices constituting about 40% of the total polypeptide chain of these proteins [41]. The amount of polypeptide chain located between the transmembrane segments of RC is substantial (about 60%). This part of the polypeptide chain protruding above the membrane surface has important structural elements with diverse types of conformations such as β-layers, β-strands, twists, and loops and includes many charged amino acids permitting electrostatic interactions with the neighboring proteins of PS-2.

It is probable that the stabilizing effect of bicarbonate is associated with its interaction with positively charged amino acid residues in the regions of these proteins protruding above the membrane surface. The most interesting of them (with regard to the literature data) are DE-loops located on the stromal (acceptor) side of the membrane, and AB-loops, CD-loops, and C-terminal domains located on the lumenal (donor) side of the membrane.

It is known that one of the signals for D1 protein aggregation is conformational changes in the stromal DE-loop [35, 39]. In addition, it has been established that the binding site of bicarbonate in PS-2 is nonheme iron located between the quinone acceptors Q_A and Q_B, which are also in the region of the DE-loop. It is likely that the affinity of bicarbonate binding with nonheme iron can be increased by electrostatic interaction between HCO_3^- and the positively charged amino acid residues in the immediate proximity to the Fe-binding site. Using point mutations, it was revealed that many conservative charged amino acid residues in DE-loops of the D1 and D2 proteins play an important role in PS-2 functioning. It has been shown that D1R269, D2R233, D2R251, D2K264, and D2R265 amino acid substitutions can influence the bicarbonate binding and functioning in PS-2 [45]. According to crystallographic analysis, the D1Y246, D2R264, and D2R265 residues are closest to the supposed bicarbonate-binding site on the acceptor side of PS-2 [2]. Substitutions for these residues result in disturbance of the electron transport from Q_A to Q_B and high concentrations of HCO_3^- ions being needed for stability of the functional activity of PS-2 [45]. It has also been shown that D1R269 and D1R257 mutations and ΔG240-V249 and ΔR225-V249 deletions in the D1 protein result in disturbance of the electron transport from Q_A to Q_B, modification of the Q_B-binding site, decrease in the oxygen-evolving activity, and the loss of DCMU binding capacity [46-48]. Nonheme iron is absent from the isolated RC; it is removed during their isolation; therefore, the main candidates for bicarbonate binding in the DE-loop are the positively charged amino acid residues. When interacting with the latter, bicarbonate can either stabilize the loop structure by preventing conformational changes resulting in protein aggregation or screen the residues directly involved in bond formation during aggregation.

It is likely that HCO_3^- ions can interact with positively charged amino acid residues also within the hydrophilic loops and *C*-terminal domains of the D1 and D2 proteins located at the lumenal surface of the membrane. It is known that certain amino acid residues (D1T342, D1E189, D1H332, D1A344, and D1H337) located on the CD-loop and in the *C*-terminal domain of the D1 protein are involved in the binding of Mn and Ca ions in the inorganic core of the WOC [2]. The lumenal loops of these proteins are supposed to participate in the interaction with the manganese-stabilizing protein PsbO that forms, together with the *C*-terminal domain of the D2 protein, a "roof" above the WOC by stabilizing the basis of conformation of the CD-loop and *C*-terminal domain of the D1 protein supplying the major ligands for components of the WOC inorganic core [2]. We demon-

strated earlier a stabilizing effect of bicarbonate on the binding of hydrophilic proteins PsbO, PsbP, and PsbQ located on the lumenal side of the PS-2 membrane [14]. In addition, it was revealed that HCO_3^- stabilizes the structure of isolated PsbO protein at acid pH values [15]. It is probable that HCO_3^- has binding sites not only at the surface of the PsbO protein as we have supposed previously [14, 15], but also in the places of close contact between this protein and the hydrophilic loops of the D1 and D2 proteins. They may include conservative positively charged amino acid residues N76, R64, N181, Q187, N192 of the D1 protein and N72, R180, and Q186 of the D2 protein [2].

The examples of specific interaction between HCO_3^- and amino acid resides of proteins are known from the literature. X-Ray structure analysis of the transporter protein CmpA (a component of ABC transport system of cyanobacteria responsible for bicarbonate transport into carboxysomes) has shown that bicarbonate liganding involves, in addition to Ca^{2+}, several amino acid residues: E271, E270, E70, Q198, N152, W99, and T192 [49]. In the work of Rowlett et al. [50], crystallographic analysis of two *Haemophilus influenzae* β-carboanhydrase mutants with V47A and G41A point substitutions revealed a new HCO_3^- binding site being an intermediate binding site for bicarbonate on its way from the carboanhydrase active center. It has been shown that HCO_3^- binding involves three amino acid residues (W39, R64, and Y181) located at a distance of 8 Å from the active center of the enzyme. It can be supposed that such interaction between bicarbonate and the proteins, resulting in their stabilization, occurs also in the RC of PS-2.

It is also probable that the revealed stabilizing effect of bicarbonate on PS-2 can be associated not only with the interaction between bicarbonate and the amino acid residues of RC proteins, but also with its ability to "quench" radicals that may form under anaerobic conditions in the course of thermoinactivation [51]. This possibility is suggested by data showing that HCO_3^- in aqueous medium can be activated by heat (40-60°C) and act as electron donor [52].

Thus, the protective effect of bicarbonate during dark thermoinactivation of isolated D1/D2/cytochrome b_{559} complex of PS-2 manifests itself both in stabilization of their photochemical activity and in prevention of the aggregation of protein components. HCO_3^- is supposed to specifically interact with the positively charged amino acid residues of hydrophilic regions of the D1 and D2 proteins, preserving the integral structure of these regions and preventing their structural modification, which is a signal for aggregation of these proteins and the loss of photochemical activity during thermoinactivation.

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