Biochimica et Biophysica Acta, 1057 (1991) 215–222

ADONIS 000527289100096G

BBABIO 43361

The catalytic role of subunit IV of the cytochrome b_6-f complex from spinach chloroplast

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(Received 12 October 1990)

Key words: Cytochrome b_6f complex; Trypsin; Spinach chloroplast

The catalytic role of subunit IV, the M_r 17000 protein, in the chloroplast cytochrome b_6-f complex was established through trypsinolysis of the complex under controlled conditions. When purified chloroplast cytochrome b_6-f complex, 1 mg/ml, in 50 mM Tris-succinate buffer (pH 7.0) containing 1% sodium cholate and 10% glycerol is treated with 80 μg of trypsin at room temperature for various lengths of time, the activity of the cytochrome b_6-f complex decreases as the incubation time increases. A maximal inactivation of 80% is reached at 7 min of incubation. The trypsin inactivation is accompanied by the destruction of the proton translocation activity of the complex. No alteration of absorption and EPR spectral properties was observed in the trypsin-inactivated complex. Subunit IV is the only subunit in the cytochrome b_6-f complex that is digested by trypsin, and the degree of digestion correlates with the decrease of electron transfer activity. The binding of azido-Q to subunit IV of the complex decreases as the extent of inactivation of the cytochrome b_6-f complex by trypsin increases. The residue molecular mass of trypsin cleaved subunit IV is about 14 kDa, suggesting that the cleavage site is at lysine 119 or arginine 125 or 126. When the thylakoid membrane was assayed for cytochrome b_6-f complex activity, very little activity was observed; and the activity was not sensitive to trypsinolysis. Upon sonication, activity and sensitivity to trypsinolysis was greatly increased, suggesting that subunit IV protrudes from the lumen side of the membrane.

Introduction

The cytochrome b_6-f complex, which catalyzes electron transfer from plastoquinol to plastocyanin in the noncyclic photosynthetic electron transport system of chloroplasts, has been shown to be functionally and structurally similar to mitochondrial cytochrome b-c_1 complex by Nelson and Neumann in 1972 [1]. Although partially purified, active cytochrome b_6-f complex preparations were obtained in 1976 [2], the pure, active preparations were not available until 1981 [3]. Since then, several improved methods for isolation of the cytochrome b_6-f complex have been introduced [4–6]. Pure cytochrome b_6-f complex contains for polypeptides with molecular masses of 33, 23.5, 19.5 and 17 kDa. These subunits house cytochrome f [7], cytochrome b_6 [8], iron-sulfur cluster [9,10] and plastoquinone (PQ) [11], respectively. The most active cytochrome b_6-f complex preparation reported had a turnover number of 20–35 per s [6], which is lower than that of mitochondrial or bacterial cytochrome b-c_1 complex. The reason for this low electron transfer activity is unknown.

Although the cytochrome b_6-f complex resembles the cytochrome b-c_1 complex in function and redox components, it differs from the latter in the redox properties of its cytochromes, lipid composition, electron acceptors, and sensitivity to electron transfer inhibitors. Both high and low-potential cytochromes b_6 in the cytochrome b_6-f complex have more negative E_m values than those of corresponding bacterial or mitochondrial cytochromes b. Cytochrome f has an E_m about 100 mV more positive than that of bacterial or mitochondrial cytochrome c_1 [7]. Phospholipid accounts for more than 90% of the total lipid in the bacterial or mitochondrial cytochrome b-c_1 complex but is only a minor component of the chloroplast cytochrome b_6-f complex [12,13], where the major lipid is glycolipid. The electron accep-

Abbreviations: 3-CHMDB, 3-chloro-5-hydroxyl-2-methyl-6-decyl-1,4-benzoquinone; DMG, decanoyl-*N*-methylglucamide; PQ, plastoquinone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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tor for bacterial b-c_1 complex is a heme protein (cytochrome c), whereas the acceptor for the cytochrome b_6-f complex is a copper protein (plastocyanin). The cytochrome b_6-f complex behaves like the cytochrome b-c_1 complex towards most electron transfer inhibitors [14]. The exceptions are antimycin and the recently discovered 3-chloro-5-hydroxy-2-methyl-6-decyl-1,4-benzoquinone (3-CHMDB) [15]. 3-CHMDB blocks electron transfer from ubiquinol to cytochrome c in mitochondrial cytochrome b-c_1 complex or from ubiquinol to cytochrome c_2 in bacterial cytochrome b-c_1 complex, but it activates electron transfer from plastoquinol to plastocyanin. These differences suggest that the oxidation mechanisms of ubiquinol and plastoquinol in their respective enzyme complexes are not identical.

The molecular structure of cytochrome b_6 has been extensively studied and recently reviewed by Cramer et al. [16]. The amino acid sequence of cytochrome b_6, deduced from gene sequence, was reported to be similar to the N-terminal portion of mitochondrial or bacterial cytochrome b [17]. The two b hemes of cytochrome b_6 are liganded with a pair of histidine residues from transmembrane helices II and V [17,18] or II and IV [19]. The observation that the two b_6 cytochromes in the cytochrome b_6-f complex were spectrally less distinguishable than cytochromes b-565 and b-562 in the mitochondrial system is explained by the more identical environment of the two hemes b in cytochrome b_6-f, than in the cytochrome b-c_1 due to the absence of one amino acid residue, Thr-184, in cytochrome b_6. Although the evidence for electron transfer between two b_6 cytochromes in the b_6-f complex is rather controversial [16,20], replacement of Phe-89 with Met in cytochrome b_6 may interrupt electron transfer between these two b cytochromes. The molecular mass of cytochrome b_6 is much smaller than that of the mitochondrial or bacterial cytochrome b. A portion of the polypeptide at the C-terminal is believed to have been cleaved and become subunit IV.

The observation of an H^+/e^- ratio of 2 in b_6-f complex embedded in phospholipid vesicles, the kinetic data on the oxidant-induced reduction of cytochrome b_6, and the presence of two PQ-binding sites indicated by inhibitor binding studies [21] suggest that the Q-cycle [22,23] is functioning in the cytochrome b_6-f complex. On the other hand, the lack of electron transfer between the two b_6 cytochromes and the insensitivity of the complex to antimycin treatment led Cramer et al. [16] to question the operation of the Q-cycle in this complex and to introduce a 'half Q cycle mechanism'. Further investigation on the structure–function relationship of the cytochrome b_6-f complex is needed before the electron transfer pathway and mechanism can be understood.

Among the four protein subunits of the complex, only subunit IV, the 17 kDa protein, bears no redox prosthetic groups. Recently, through the study of photoaffinity labeling using azidoquinone derivatives, it has been established that the 17 kDa protein serves as a plastoquinone binding site for the complex [11]. In contrast to cytochrome b-c_1 systems, cytochrome b_6 does not bind quinone, suggesting that the quinone-binding site in the cytochrome b_6-f complex differs from that in cytochrome b-c_1 complexes. More recently, we have confirmed, as expected, that the intactness of the 17 kDa protein is catalytically essential for electron transfer. When the cytochrome b_6-f complex is subjected to limited trypsinolysis under conditions in which only the 17 kDa protein is affected, the decrease in electron transfer activity is directly correlated with the destruction of the 17 kDa protein. In this paper we report the detailed conditions for trypsinolysis and the characterization of the trypsin-inactivated cytochrome b_6-f complex. Preliminary results have been reported [25].

Materials and Methods

Lipid and plastoquinone-deficient cytochrome b_6-f complexes were prepared and assayed by the method described previously [11]. The preparation showed only partial activity. Full activity was obtained after reconstitution with plastoquinone and asolectin or lipid prepared from chloroplast. Intact thylakoid membrane was prepared according to Zhou [26]; the inside-out membrane was prepared by a 2×2 min sonication, using a Heat-System Ultrasonics' sonicator, model W-220F, with a microtip at a power output setting of 2.

Azido-Q derivatives [27], plastoquinone-2 [28], plastoquinol-2 [28] and DMG [29] were synthesized according to reported methods. Cytochrome c, type III, trypsin, trypsin, inhibitor, and sodium cholate were purchased from Sigma. Other chemicals used were of the highest purity commercially available.

Trypsinolysis

The cytochrome b_6-f complex, 1 mg/ml, in 50 mM Tris-succinate buffer (pH 7.0) containing 1% sodium cholate and 10% glycerol, was treated with a given amount of trypsin (0 to 0.24 mg/mg b_6-f protein) at room temperature for various time periods. Proteolysis was stopped either by the addition of a 10 molar excess of trypsin-inhibitor or the addition of 2% SDS. In the control experiment, trypsin inhibitor or SDS was added before trypsin. For activity assay of the trypsin-treated complex, the mixture was diluted to 0.2 mg/ml with 50 mM Tris-succinate buffer (pH 7.0) containing 1% sodium cholate and 1.25 mg/ml spinach chloroplast lipid. This mixture was then incubated at 0 °C for 30 min before being assayed. 100% activity is 35 μmol cytochrome c reduced/nmol cytochrome f per h at room temperature, which is the activity of the control

sample or the sample without trypsin treatment after reconstitution with lipids.

Preparation of antibodies against a decapeptide of subunit IV

A polypeptide composed of 10 amino acid residues (PLEILPEWYF), corresponding to residue numbers 72 through 81 of subunit IV, was synthesized, purified, and used as an antigen to raise antibodies from rabbits. The synthetic peptide was coupled to ovalbumin using glutaraldehyde [30], dialyzed in 50 mM sodium/potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl, and mixed with Freund's complete adjuvant (1 : 1). A rabbit was injected subscanpularly with 1 mg peptide weekly for 2 weeks. A booster was given at the end of the 4th week. Serum was collected by cardiac puncture 1 week after the booster.

Incorporation of azido-Q derivative into trypsin cytochrome b_6-f complex

The conditions for incorporation and distribution of azido-Q in the subunits of the cytochrome b_6-f complex were the same as those described previously [11]. 30 molar excess of azido-Q was used. Azido-Q was added at the end of trypsinolysis after the addition of trypsin inhibitor. The azido-Q was allowed to react with the protein for 20 min before being illuminated with long-wavelength ultraviolet light for 7 min at 2°C. Subsequent steps were the same as described before [11].

The cytochrome b_6-f complex phospholipid vesicles were prepared essentially according to the method of by Leung and Hinkle [24], except a phospholipid-to-protein ratio of 30 was used.

Spectral measurements were carried out in a SLM-Aminco double-beam, dual-wavelength spectrometer, model DW-2000, at room temperature. EPR measurements were performed in a Bruker ER-200 D spectrometer, equipped with an Oxford liquid helium system.

Results and Discussion

Inactivation of the cytochrome b_6-f complex by trypsinolysis

Fig. 1 shows trypsin concentration-dependent inactivation of the cytochrome b_6-f complex. When cytochrome b_6-f complex, 1 mg/ml, in 50 mM Tris-succinate buffer (pH 7.0) containing 1% sodium cholate and 10% glycerol, is treated with increasing concentrations of trypsin for 7 min at room temperature, the plastoquinol-cytochrome c reductase activity decreases as the concentration of trypsin increases. A maximal inactivation of 75% is reached when 80 μg of trypsin per mg of the b_6-f protein is used. Under identical conditions, the control experiments (either in the absence of trypsin or when the trypsin inhibitor is added prior to the addition of trypsin), show less than 5% inactivation.

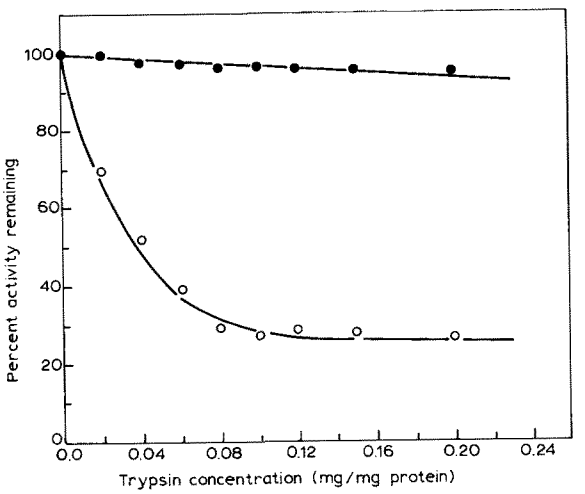

Fig. 1. Trypsin concentration-dependent inactivation of the cytochrome b_6-f complex. The cytochrome b_6-f complex (10 μM cytochrome f), in 50 mM Tris-succinate buffer (pH 7.0) containing 1% sodium cholate and 10% glycerol was digested with the indicated amounts of trypsin for 7 min at room temperature (○———○). The reaction was terminated by the addition of 3-fold molar excess of soybean trypsin inhibitor. The control samples (●———●) were prepared under identical conditions except that the soybean trypsin inhibitor was added to the complex prior to the addition of trypsin. For activity assays, the digested samples were diluted to 2 μM cytochrome f with 50 mM Tris-succinate (pH 7.0) containing 1% sodium cholate and 1.25 mg/ml spinach glycolipids, and incubated at 0°C for 30 min. 100% activity represents 35 μmol cytochrome c reduced per nmol cytochrome f per h.

Fig. 2 shows the time-dependence of inactivation. When the cytochrome b_6-f complex is treated with 80

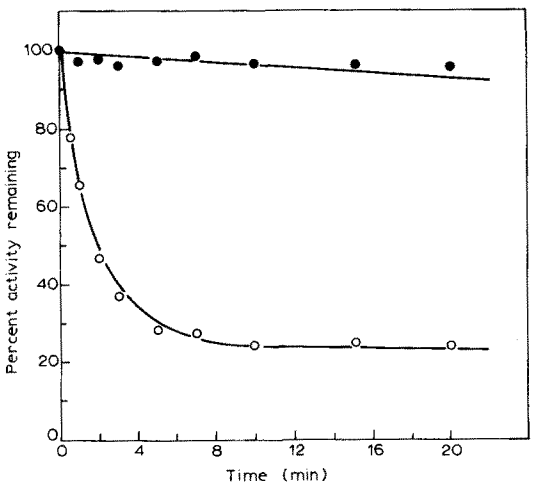

Fig. 2. Effect of trypsin digestion time on the activity of cytochrome b_6-f complex. The experimental conditions were the same as in Fig. 1, except that a constant amount of trypsin (80 μg/mg) complex was used and incubation times were varied. The curve with open symbols (○) shows the sample treated with trypsin, and the curve with closed (●) symbols represents the control experiments in which the soybean trypsin inhibitor was added to the complex before the addition of trypsin.

μg trypsin per mg of the b_6-f complex protein and incubated at room temperature for various lengths of time, 50% inactivation requires less than 2 min of incubation. Maximal inactivation is reached at 7 min incubation. Again, when trypsin inhibitor (100-fold molar excess) is added prior to the addition of trypsin, little inactivation is observed after 30 min incubation.

Characterization of trypsin-treated cytochrome b_6-f complex

The cytochrome b_6-f complex contains four subunits: subunit I (cytochrome f) with a molecular weight of 33000; subunit II (cytochrome b_6) with a molecular weight of 23000; subunit III (non-heme iron sulfur protein) of 21500; and subunit IV (Q-binding protein), of 17000. It is important to known which subunit(s) is (are) responsible for the loss of plastoquinol-cytochrome c reductase activity during trypsin treatment. One way to locate the target site is to examine the physical properties and protein structures of the subunits in the trypsin-treated cytochrome b_6-f complex and compare them to those in untreated samples.

Cytochromes f and b_6

Fig. 3 compares the absorption characteristics of cytochromes f and b (subunits I and II) in the cytochrome b_6-f complex with (panel B) and without (panel A) trypsin treatment. Although the trypsin-treated complex has less than 20% of the activity of the untreated, virtually no changes in the absorption characteristics of cytochromes b_6 and f is observed after trypsinolysis. This suggests that either cytochromes b_6 and f in the b_6-f complex are not susceptible to trypsin digestion or the cleavage of peptide bonds by trypsin does not alter the tertiary structure, and hence the spectral properties, of these cytochromes. The second explanation has been ruled out by the fact that SDS-PAGE shows no change in the molecular weights of cytochromes b_6 and f during trypsinolysis. It is likely that either lipids in the complex that are strongly associated with these cytochromes protect them from trypsin, or the cytochromes are arranged in such a way that no susceptible lysine or arginine residue are exposed. Szczepaniak et al. [31] observed some cleavage of cytochrome b_6 upon trypsinolysis of thylakoid membrane under harsher conditions.

Iron sulfur protein

A high-potential [2Fe-2S] protein, generally known as Rieske's protein (subunit III), which exhibits an unique EPR signal at $g = 1.90$, is another important redox component in the cytochrome b_6-f complex. Trypsin treatment did not change the EPR spectra of the iron-sulfur clusters (Fig. 4). This suggests that trypsinolysis of the cytochrome b_6-f complex does not damage the iron-sulfur protein. This is substantiated by

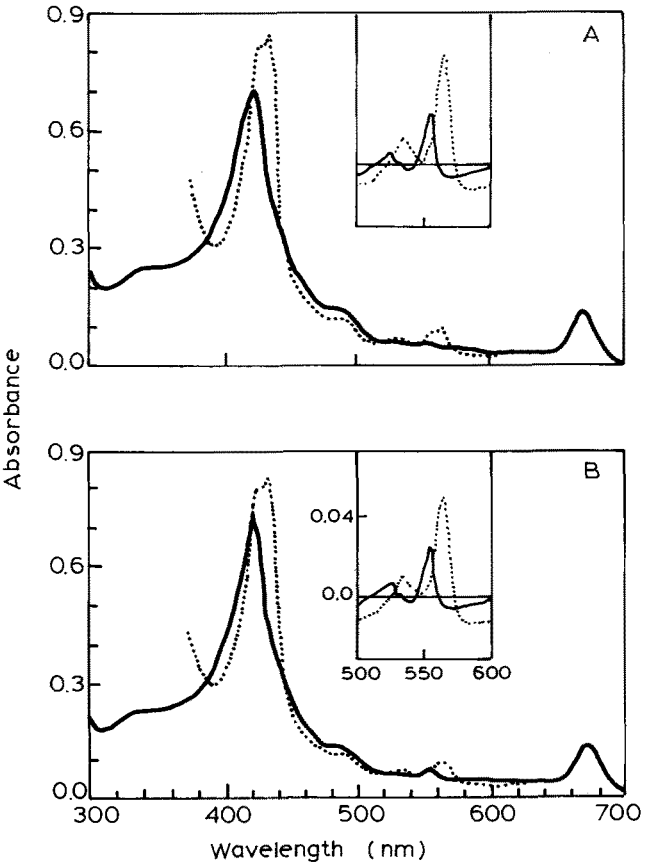

Fig. 3. Effect of trypsin digestion on the spectral properties of cytochrome b_6-f complex. Cytochrome b_6-f complex (10 μM cytochrome f) was treated with 80 μg trypsin/mg protein for 0 min (A) or 7 min (B). The reaction of trypsin was terminated by the addition of inhibitor. After treatment, the samples were diluted to 2 μM cytochrome f with 50 mM Tris-succinate (pH 7.0) containing 1% sodium cholate. Solid curves (———) represent the treated cytochrome b_6-f complex and the dashed curves (— — —) are the sodium dithionite-reduced samples. The insets are difference spectra of ascorbate reduced minus ferricyanide oxidized samples (———) and the sodium dithionite-reduced minus ascorbate-reduced samples (— — —).

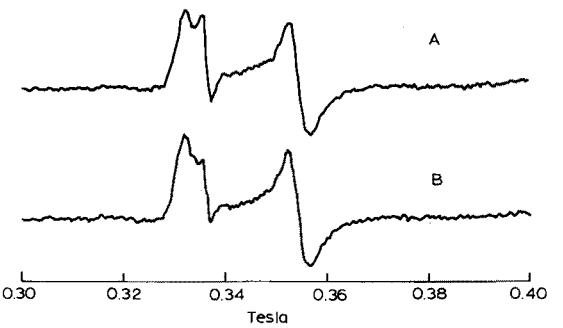

Fig. 4. Effect of trypsin treatment on the iron-sulfur protein in the cytochrome b_6-f complex. Cytochrome b_6-f complex, 1 mg/ml, was treated with 80 μg trypsin/mg complex for 7 min. The samples were concentrated to 20 mg/ml and reduced with ascorbate before subjection to EPR analysis at 80 K. The instrument settings were as follows: field modulation frequency, 100 kHz; microwave frequency, 9.34 GHz; modulation amplitude, 6.3 G; microwave power, 20 mW.

the observation that the polypeptide structure of the iron-sulfur protein is not altered during trypsinolysis (see Fig. 5).

Subunit IV

Since the three larger subunits, which house cytochromes *f* and b_6, and iron-sulfur cluster have been ruled out as the destruction site for trypsin, subunit IV (plastoquinone-binding subunit) is the likely candidate for inactivation by trypsin. Subunit IV has been identified as the plastoquinone binding protein by photoaffinity labeling techniques using azido-Q derivatives. Unlike heme and iron-sulfur cluster, determination of the destruction of subunit IV through spectral analysis is difficult. One way to follow the effect of trypsinolysis on subunit IV is to monitor the change of its molecular size by SDS-PAGE. Fig. 5 shows SDS-PAGE patterns of the cytochrome b_6-*f* complex treated with trypsin for various time periods. When the cytochrome b_6-*f* complex is treated with trypsin, the 17 kDa protein (subunit IV) decreases as incubation time increases. This decrease of the 17 kDa protein correlates with the activity loss. Since cytochromes b_6, and *f* and iron-sulfur protein are not affected by trypsin treatment and the 17 kDa protein is digested, it is clear that inactivation of the complex is due to destruction of the 17 kDa protein. Thus, the 17 kDa protein is catalytically essential in this complex. Until the recent identification of the 17 kDa protein as a PQ-binding protein, the role of this protein was in doubt and thought to be a regulatory one.

The 17 kDa protein is first cleaved to become 14 kDa; as incubation time is lengthened, the 14 kDa segment is further degraded. This is evident because the loss of 17 kDa protein correlates with the appearance of a new protein band at approx. 14 kDa (Fig. 6) after prolonged incubation, a slight decrease in the fragment of 14 kDa is observed. Examinations of the amino acid sequence reported for the 17 kDa protein [32,33] suggests that the peptide bond cleaved by trypsin to generate a 14 kDa fragment is either arginine 125, 126 or lysine 119. If the amino acid residue numbering system * of Cramer et al. [16] for the 17 kDa protein is used, the numbers of these amino acid residues would be arginine 318, 319 or lysine 312. As described in a previous section, the 17 kDa protein is believed to be the truncated portion of the C-terminal end of cytochrome b_6. The amino acid numbering at the N-terminal starts with 220 instead of 1 in order to align it with other cytochrome *b* protein sequences [16]. The portion that is cleaved off by trypsin in the 17 kDa protein corresponds to the last transmembrane helix of the protein. If the predicted transmembrane arrangement [33] is correct, the trypsin cleavage site is on the same side as the N-terminal, the side opposite the C-terminal. The trypsin cleavage site will be on the lumen side of the thylakoid membrane.

To further substantiate that the 14 kDa protein is derived from subunit IV and not from another subunit, antibodies against a decapeptide of subunit IV were raised from rabbits and used to identify the origin of the 14 kDa protein using the Western blotting technique. Fig. 5B shows the Western blotting of the intact and the trypsin treated cytochrome b_6-*f* complex using antibodies against a peptide of subunit IV. Both subunit IV and the 14 kDa protein react to the antibodies, suggesting that the 14 kDa protein is indeed derived from subunit IV. It should be mentioned here that antibodies against the decapeptide of subunit IV also react with cytochrome *f*. Although this does not pose any complication in the identification of the origin of the 14 kDa protein as cytochrome *f* was not degraded by the treatment of trypsin under the experimental conditions employed, some explanation is needed as to why the antibodies react to cytochrome *f*. Since neither the synthetic decapeptide nor ovalbumin possesses any amino acid sequence homologous to the that of cytochrome *f*, the epitope or epitopes on cytochrome *f* must be conformational rather then sequential. If this explanation is correct, than the decapeptide region of subunit IV must interact closely with cytochrome *f*. Further experimentation is needed before this speculation can be confirmed.

Effect of azido-Q binding to the 17 kDa protein upon trypsinolysis of the cytochrome b_6-f complex

Since the 17 kDa protein serves as the PQ binding site in the complex, it is important to know whether trypsinolysis of the complex affects PQ binding. This is investigated by comparing the incorporation of azido-Q into the 17 kDa subunit of the cytochrome b_6-*f* complex with and without trypsin treatment. When the complexes are treated with trypsin for different lengths of times prior to photoaffinity labeling with [H]azido-Q, the amount of azido-Q incorporation into the cytochrome b_6-*f* protein decreases as the complex becomes progressively inactivated. When these azido-Q treated, trypsin digested cytochrome b_6-*f* complexes are subjected to SDS-PAGE, incorporation of azido-Q into the 17 kDa protein decreases as incubation time increases (see Fig. 7). The decrease of the radioactivity in the 17 kDa protein correlates with the increase of radioactivity in the 14 kDa protein and in lower-molecular mass fragments. Some radioactivity appears in a low-molecular mass fragment undetectable by protein staining. This probably results from the nonspecific binding of peptides truncated from the 17 kDa protein because the azido-Q derivative is introduced after the end of trypsinolysis. The trypsinolysis of cytochrome b_6-*f* complex

* The numbering system of amino acid residues of subunit IV is slightly different between laboratories.

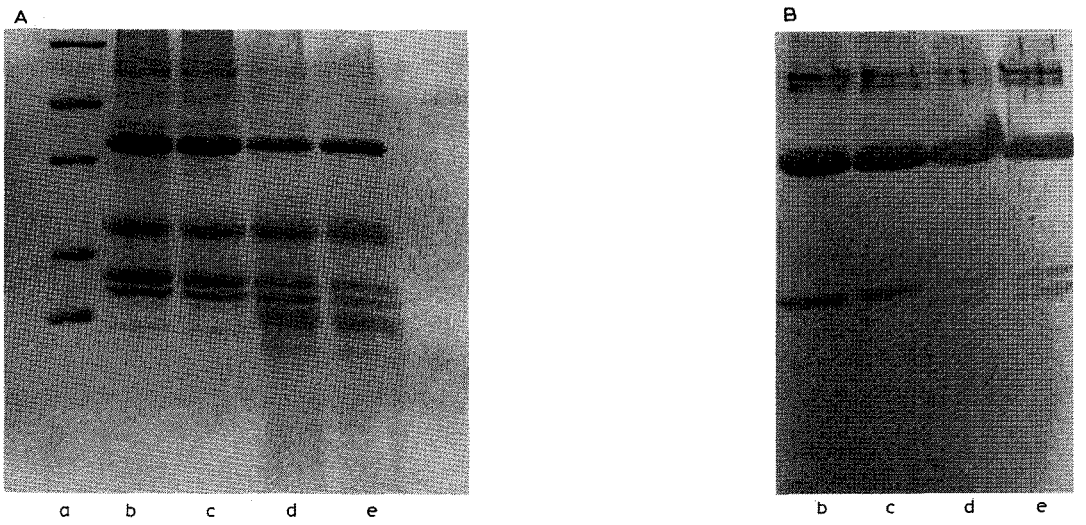

Fig. 5. SDS-PAGE and Western blotting of the intact and trypsin-treated cytochrome b_6-f complexes. (A) SDS-PAGE, cytochrome b_6-f complex, 1.1 mg/ml in 50 mM Tris-succinate buffer (pH 7.8) containing 1% sodium cholate and 10% glycerol, was digested with 80 μg trypsin per mg protein for 0 (c), 5 (d) and 15 (e) min at room temperature. The reaction was terminated by the addition of 2% SDS and 1% β-mercaptoethanol. The mixtures were incubated at 37°C for 2 h before being subjected to SDS-PAGE. Lane a displays the molecular mass standard proteins (phosphorylase, 97 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 21 kDa; and lysozyme, 14 kDa), and lane b is the intact cytochrome b_6-f complex. (B) Western blotting was carried out using antibodies against a synthetic decapeptide of subunit IV. Samples of (A), except for the molecular mass standard proteins, were subjected to SDS-PAGE and transferred to a nitrocellulose membrane electrophoretically without staining. The membrane was then treated with anti-decapeptide before reacting with goat anti-rabbit IgG-alkaline phosphatase conjugate. The detection of alkaline phosphatase activity was performed using a color development kit (Bio-Rad).

is stopped by the addition of inhibitor. The labeling that appears in the 14 kDa fragment correlates with the amount of this fragment present in samples incubated with trypsin. This suggests that the 14 kDa fragment is capable of binding PQ, at least to a certain degree. The nature of the binding, however, is difficult to assess. Apparently, it is not the same as in the intact 17 kDa protein because catalytic activity is lost in the treated samples. A careful examination of the PQ radical on the intact and trypsin-treated complexes may yield information useful in reaction mechanism studies and PQ binding site elucidation. Investigation of this aspect is currently in progress.

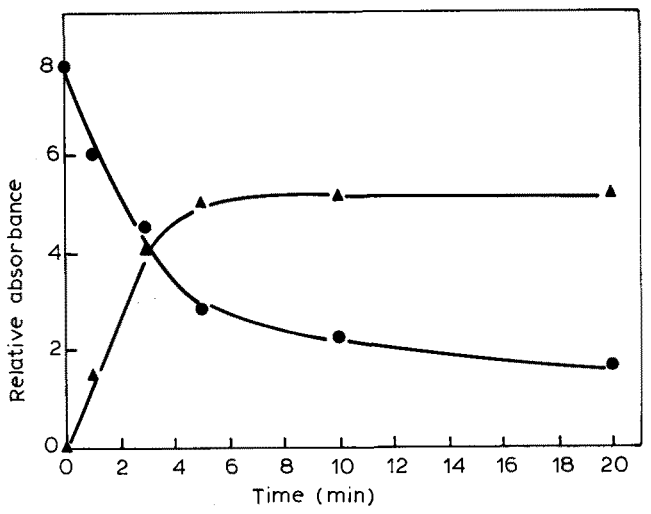

Fig. 6. Correlation between the decrease of the 17 kDa protein and the increase of the 14 kDa protein during the trypsinolysis of cytochrome b_6-f complex. The concentrations of the 17 kDa and 14 kDa proteins were determined by densitometric tracing of the stained gels at 600 nm. Other experimental conditions were the same as in Fig. 5 except that the trypsin reaction was terminated by the addition of 10-fold molar excess of trypsin inhibitor instead of 2% SDS.

Effect of trypsin treatment of cytochrome b_6-f inlaid in phospholipid vesicles

If the trypsin cleavage site on the 17 kDa protein is at Arg-125, -126, or Lys-119, it is expected that this site will be exposed to the aqueous phase and susceptible to trypsin treatment when cytochrome b_6-f is reconstituted into phospholipid vesicles, because these amino acid residues are located between transmembrane segments of the 17 kDa protein predicted by hydropathy plots. However, when the cytochrome b_6-f complex is reconstituted into phospholipid vesicle and then subjected to trypsin treatment, only about 20% of the electron transfer activity is lost (Fig. 8). The untreated b_6-f-PL vesicle has a proton ejection/electron transfer ratio of 1.8, which is very close to the value reported by other investigators [34]. No change in the H^+/e^- ratio is observed in the b_6-f-PL vesicle treated with trypsin (data not shown). As expected when the vesicle is disrupted by detergent, a significant increase in electron transfer activity is observed and the degree of inactivation by trypsin treatment increases. Apparently, the major cytochrome b_6-f complex molecules are oriented with the 17 kDa protein facing inward, similar to the arrangement

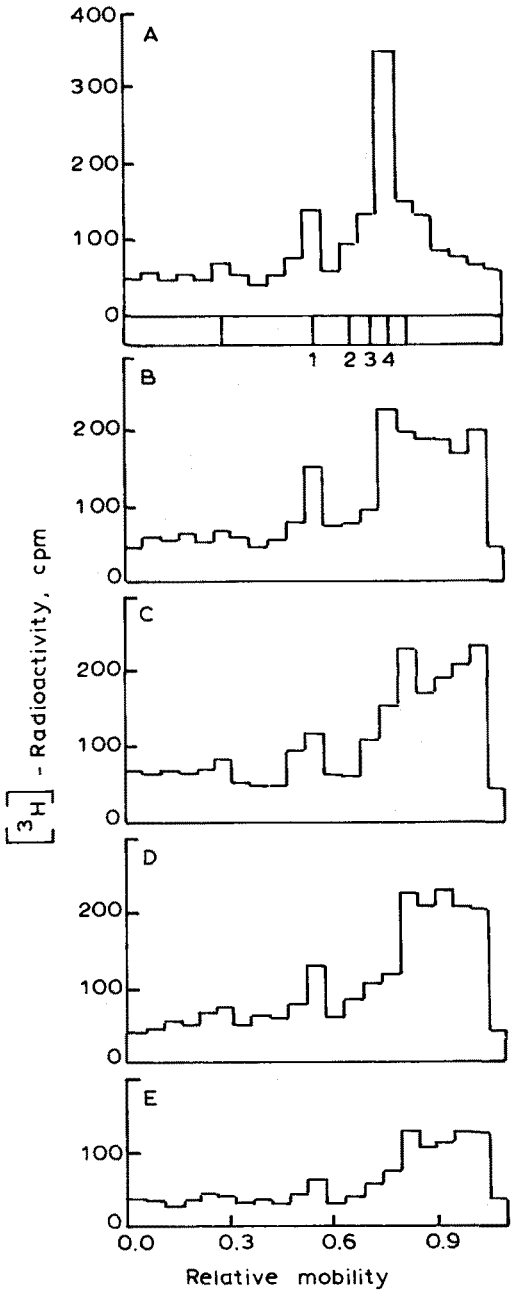

Fig. 7. Effect of trypsin treatment on the distribution of photoaffinity labeling of cytochrome b_6-f complex by azido quinone. Cytochrome b_6-f complex was treated with trypsin under the same conditions given in Fig. 3. After the trypsin reaction was terminated by the addition of inhibitor, the samples were photolyzed with 30-fold excess of azido Q for 7 min. The photolyzed samples were dialyzed, extracted with organic solvent, lyophilized and subjected to SDS-PAGE after dissociation in 50 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 1% β-mercaptoethanol. The conditions for electrophoresis and the estimation of radioactivity distribution are the same as reported previously [11]. The Arabic numerals, 1 to 4, represent subunits I to IV of the cytochrome b_6-f complex.

of the cytochrome b_6-f complex in the thylakoid membrane. This type of b_6-f complex becomes active when the vesicles are disrupted because the electron acceptor cytochrome c becomes accessible to cytochrome f. Since

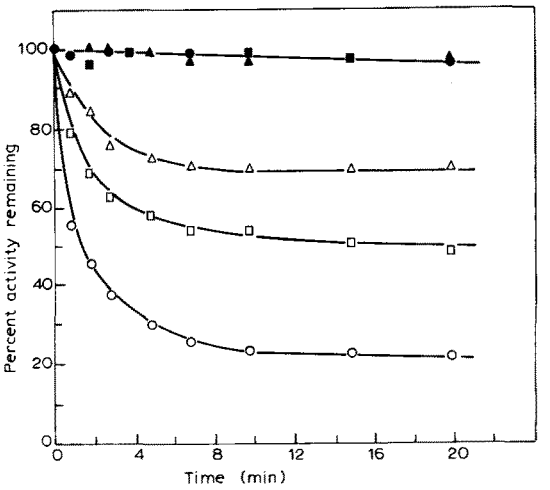

Fig. 8. Effect of trypsin treatment on cytochrome b_6-f complex inlaid in phospholipid vesicles. Cytochrome b_6-f complex-phospholipid vesicles as prepared (△, ▲), 0.5% DMG dispersed (□, ■) and purified (○, ●) complex were incubated at room temperature in the presence (△, □, ○) and absence (▲, ■, ●) of 80 μg trypsin per mg b_6-f complex. The concentration of cytochrome b_6-f complex was 10 μM, based on cytochrome f. 100% activity represents 11, 35 and 31 μmol cytochrome c reduced per nmol cytochrome f per h for vesicles, dispersed vesicles and isolated complex preparations, respectively.

the degree of inactivation of plastoquinol cytochrome c reductase activity by trypsinolysis differs significantly between the vesicles and the detergent-dispersed vesicles, the exposure of trypsin susceptible sites must differ in these two preparations.

When isolated intact thylakoid membrane is subjected to trypsinolysis, very little plastoquinol cytochrome-c reductase activity is lost (see Fig. 9), indicat-

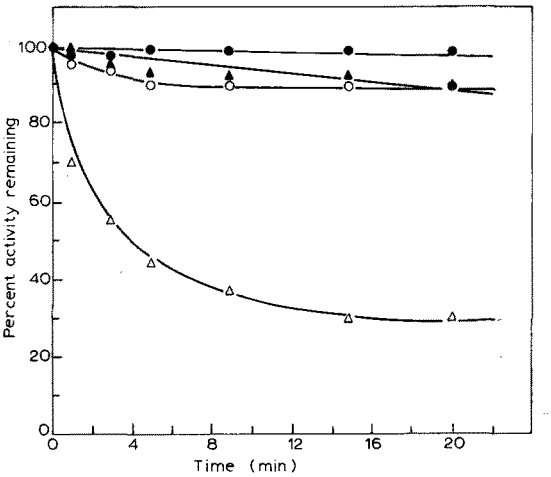

Fig. 9. Effect of trypsin digestion on the plastoquinol-cytochrome c reductase activity of thylakoid membranes. Thylakoid membranes, prepared (○, ●) and sonicated (△, ▲), were incubated at room temperature in the presence (○, △) and absence (●, ▲) of trypsin. The concentration of trypsin used was normalized to the content of the cytochrome b_6-f complex present in the membrane. Other experimental conditions are the same as those given in Fig. 9. The 100% activity represents 5 and 11 μmol of cytochrome c reduced per mole cytochrome f per h as prepared and sonicated.

ing that the trypsin-susceptible site of the 17 kDa protein is not located on the stroma side. When the thylakoid membrane is subjected to sonication before determination of plastoquinol cytochrome *c* activity, a 10-fold increase is observed, suggesting the formation of an inside-out or leaky membrane during sonication. When the sonicated preparation is treated with trypsin, a drastic decrease in activity is observed. These results suggest that the trypsin cleavage site of the 17 kDa proteins is located on the lumen side of the thylakoid membrane. Since the N-terminal of the 17 kDa protein is two transmembrane segments from the the trypsin cleavage site, it must also be located on the lumen side. The C-terminal, which is only one transmembrane segment away from the trypsin cleavage site, is therefore on the stroma side. Further confirmation of this topological arrangement must await immunobinding analysis using antibodies against different segments of the 17 kDa subunit. This work is in progress.

Acknowledgements

This work was supported by grants from the NIH (GM 30721) and the USDA (87-CRCR-1-2433). We thank Dr. S. Usui for preparation of nonspecific polyclonal antibodies and Dr. Roger Koeppe for critically reading the manuscript.

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