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Fluorescence emission spectra and thylakoid protein kinase activities of three higher plant mutants deficient in chlorophyll *b* *

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The properties of three higher plant mutants having less than normal amounts of chlorophyll *b* were compared with their respective wild-types. These mutants included the chlorophyll-*b*-lacking U374 sweet clover (*Melilotus alba*) and chlorina-*f*2 barley (*Hordeum vulgare*) as well as the chlorophyll *b*-deficient CD3 wheat (*Triticum aestivum*). Fluorescence emission spectra from leaves of the sweet-clover mutant at 77 K show great similarity to the previously published spectrum of the barley mutant; rather than the predominant long-wavelength emission at approx. 740 nm in the wild-type plants, an emission maximum at approx. 720 nm is observed. The wheat mutant, containing reduced but measurable amounts of chlorophyl *b*, had 77 K long-wavelength fluorescence emissions at both 720 and 740 nm. These data indicate that these PS-I-derived fluorescence emissions are strongly influenced by the presence of antennae components. When examined for the ability to perform a light-induced State 1–State 2 transition in vivo, none was detected in the U374 sweet clover, whereas the CD3 wheat was capable of this process. The phosphorylation of endogenous polypeptides in isolated thylakoid membranes was examined using [γ-^{32}P]ATP as substrate for the thylakoid protein kinase activities. All three mutants had higher thylakoid protein kinase activity than the respective normal plants on a chlorophyll basis. The response of the mutant and normal sweet clover thylakoid protein kinase activities to ATP concentration was essentially identical. In contrast, the thylakoid protein kinase activities in the barley and wheat mutants appeared to saturate at markedly lower ATP concentrations than in the respective normal plants. These data suggest that the chlorina-*f*2 and CD3 mutants may be lacking one of the thylakoid protein kinases normally present in wild-type plants and that mutants lacking chlorophyll *b* may be of at least two different types.

Introduction

Much of our information and ideas about the organization of the photosynthetic apparatus of higher plants is derived from experiments using mutants lacking Chl *b*. The chlorina-*f*2 mutant of barley (*Hordeum vulgare*) has been the most frequently used of the available mutants (e.g. Refs. 1–5). One area of photosynthesis research that has received much attention recently is the effect of thylakoid protein phosphorylation on the regulation of quantal energy distribution within the photosynthetic apparatus. The current model for this phenomenon postulates that phosphorylation of LHC-2, a Chl-*b*-containing antenna pigment-protein complex, physically moves it further from PS II and closer to PS I [6–9]. A major piece of evidence supporting this hypothesis is that the ability to regulate quantal distribution, termed the State 1–State 2 transition [2], appears to be

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Abbreviations: Chl, chlorophyll; PS I, Photosystem I; PS II, Photosystem II; LHC-2, the light-harvesting chlorophyll *a*/*b*-protein complex dedicated primarily to PS II.

absent from the chlorine-*f*2 mutant of barley [4,5] which lacks LHC-2 [1]. It has also been reported that the amount of thylakoid protein kinase activity in the chlorine-*f*2 mutant is greatly reduced relative to wild type [4].

In this report we examine two additional mutants either lacking or deficient in Chl *b* and compare their thylakoid protein kinase activities and fluorescence emission spectra at 77 K to those in the chlorina-*f*2 mutant of barley. These data appear to indicate that these mutants contain more thylakoid protein kinase activity than the wild-type plants, and that some mutants lacking or deficient in Chl *b* may also be deficient in at least one of the thylakoid protein kinase activities.

Materials and Methods

Plants were grown in a glasshouse in a soil-vermiculite mixture for approximately four weeks prior to use. Seeds of normal (U389) and mutant (U374) sweet clover (*Melilotus alba*) were the gift of Drs. H.J. Gorz, USDA-ARS, and F.A. Haskins, Department of Agronomy, University of Nebraska, Lincoln. Seeds of normal (Lyon) and mutant (chlorina-*f*2) barley (*Hordeum vulgare*) were the gift of Dr. Harry Highkin, California State University. Seeds of normal (ND-496) and mutant (CD3) wheat (*Triticum aestivum*) were the gift of Dr. Murray Duysen, Botany Department, North Dakota State University.

Thylakoid membranes were isolated as described previously [10]. Chlorophyll concentrations and the ratio of Chl *a*/*b* were determined using the method of Arnon [11]. Wild-type plants normally had measured ratios of Chl *a*/*b* of 2.7–3.0. The CD3 wheat plants usually had ratios of Chl *a*/*b* of 6–7, whereas the U374 and chlorina-*f*2 mutants had ratios in excess of 20, above the valid range of the assay. Protein concentrations were determined by a modification [12] of the method of Lowry et al. [13], except that sample absorbance was determined at 750 nm to avoid interference from chlorophyll. The ability of thylakoids in vivo to perform a State 1–State 2 transition at 20° was determined as previously described [5] from the kinetics of LHC-2/PS II modulated fluorescence emission (685 nm) observed upon exposure of leaf tissue that was being irradiated with modulated blue light, to additional excitation with far red light (710 nm) absorbed preferentially by PS I. The balance of intensities of the blue and red irradiations is crucial to the observation of such a State 1–State 2 transition in these experiments. Hence, when using mutant plants with different absorption characteristics, the balance of excitation levels was altered to ensure that any lack of a State 1–State 2 transition was not due to an improper balance of exciting wavelengths.

Assay for thylakoid protein kinase activity was with a filter paper method [10,14], except that the assays each contained 10 μCi of [γ-^{32}P]ATP; all determinations were made in triplicate.

Fluorescence emission spectra from dark-adapted leaf tissue and isolated thylakoid membranes were determined at 77 K much as described by Dominy and Baker [15]. Samples were dark-adapted for 10 min, placed in the sample chamber containing liquid nitrogen and allowed to equilibrate for 5 min. Irradiation (435 nm, bandwidth 20 nm) was produced by a xenon source through a high irradiance monochromator (Applied Photophysics Ltd, London, U.K.) and taken to the sample chamber by one arm of a trifurcated light pipe and a 120 × 10 mm diameter quartz light guide. The photon flux density at the surface of the sample was 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Fluorescence emission from the sample was transmitted through a second arm of the light pipe and a diffraction grating monochrometer (Applied Photophysics Ltd.) with entrance and exit slits of 0.5 mm and 2.0 nm, respectively. The wavelength was changed by a stepping motor drive unit over a wavelength range from 650 to 800 nm at a rate of 120 $nm \cdot min^{-1}$. Fluorescence was detected with a Hamamatsu R928 photomultiplier tube.

Most chemicals were purchased from Sigma Chemical Co. Buffers and ATP was purchased from Calbiochem-Behring. [γ-^{32}P]ATP was purchased from New England Nuclear Corp.

Results

Chlorophyll fluorescence emission spectra were determined at 77 K using leaves from wild-type and CD3 mutant wheat (Fig. 1) and from wild type and U374 mutant clover (Fig. 2). The fluores-

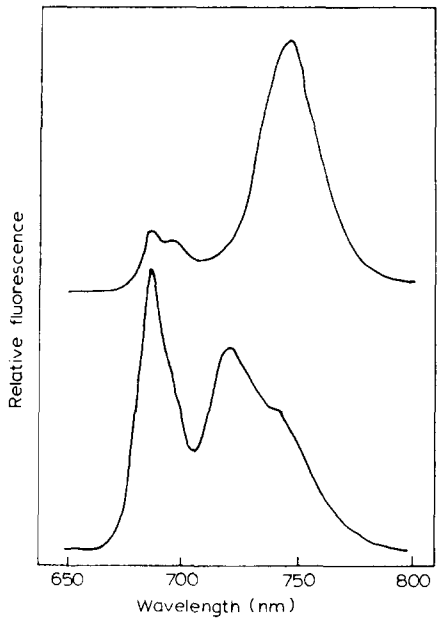

Fig. 1. Fluorescence emission spectra (77 K) of wild-type and CD-3 mutant wheat leaves. Excitation wavelength was 435 nm. Spectra: top, wild type leaves; bottom, mutant leaves.

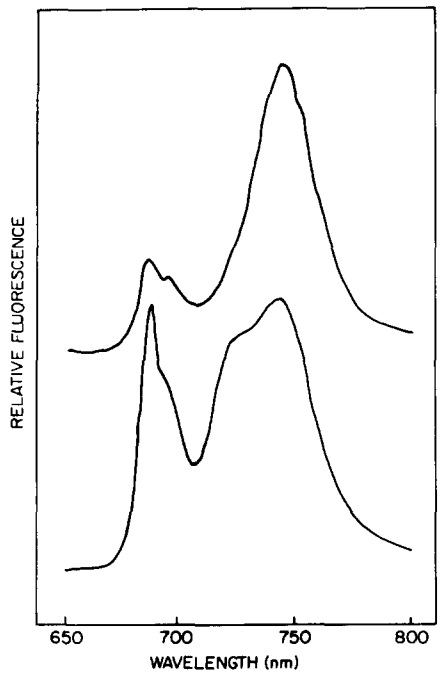

Fig. 2. Fluorescence emission spectra (77 K) of wild-type and U374 mutant sweet clover leaves. Spectra: top, wild-type; bottom, mutant leaves.

cence emission spectra of the normal leaves of both wheat and sweet clover show the characteristic emission maxima at 685, 695 and 740 nm routinely observed for most higher plants [16]. The CD3 mutant wheat, partially deficient in Chl *b* [17], has four fluorescence emission components with maxima at 685, 695, 720 and 740 nm. The U374 mutant, totally lacking in Chl *b* [18], has three readily observed components of fluorescence emission with maxima at 685, 695 and 720 nm. The 740 nm emission maximum is greatly diminished in the latter mutant.

There can be problems when interpreting fluorescence emission signals from intact leaf tissues containing different chlorophyll contents due to the possible reabsorption of fluorescence. This has the effect of enhancing the magnitude of the longer wavelength fluorescence emissions relative to those at shorter wavelengths. Because of this possibility, fluorescence emission spectra were recorded from thylakoid membranes isolated from CD3 mutant and wild-type wheat leaves and suspended at low chlorophyll concentrations (10 $\mu g \cdot ml^{-1}$). As can be seen from Fig. 3, the fluorescence emission

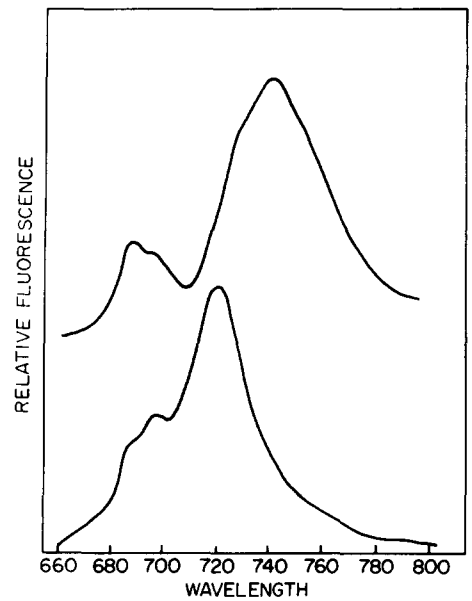

Fig. 3. Fluorescence emission spectra (77 K) of isolated thylakoid membranes from wild-type and CD-3 mutant wheat. Membranes were suspended to a Chl concentration of 10 $\mu g \cdot ml^{-1}$. Spectra: top, wild-type membranes; bottom, mutant membranes.

spectra of the CD3 mutant wheat thylakoids still contain the four components of fluorescence emission at 685, 695, 720 and 740 nm, as observed from the intact leaf tissue (Fig. 1), although the relative magnitudes of the peaks are different. In fact, the ratio of 740 to 720 nm fluorescence is greater in the diluted thylakoids than in the leaves, probably reflecting the change in the environment around the membranes. This demonstrates that self-absorption artifacts do not hinder the identification of chlorophyll fluorescence emission components from intact leaf tissue, and that low-temperature fluorescence emission spectroscopy can be a valid and useful technique for the rapid analysis of pigment-deficient mutants.

Thylakoid protein kinase activity was found in the wild-type and mutants of all three species. In addition to assaying the amount of activity present, the response of the activities to ATP concentration was examined. The wild-type and U374 mutant of sweet clover had essentially identical responses of thylakoid protein kinase activity to ATP concentration (Fig. 4). The actual values for kinase activity at 0.8 mM ATP were 66 and 220 $pmol \cdot min^{-1} \cdot mg^{-1}$ of Chl for the wild-type and U374 mutant, respectively. In contrast, the chlorina-*f*2 barley (Fig. 5A) and the CD3 wheat (Fig. 5B) thylakoid protein kinase activities responded

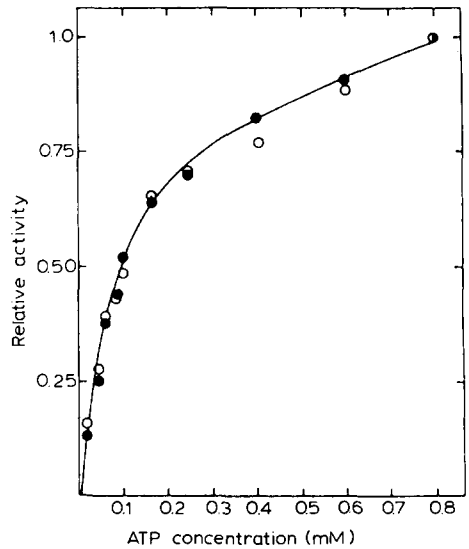

Fig. 4. Response of wild-type and U374 mutant sweet clover thylakoid protein kinase activities to ATP concentration. Symbols: (●) wild-type; (○) mutant.

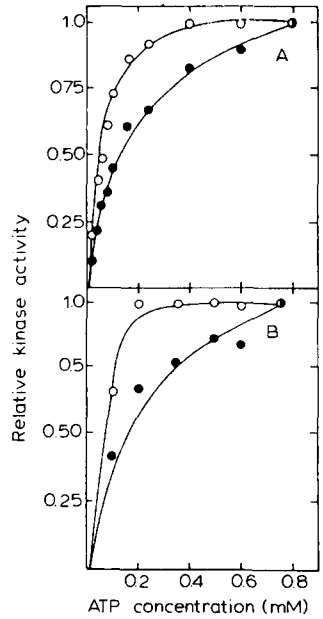

Fig. 5. Response of thylakoid protein kinase activity to ATP concentration in barley and wheat. (A) ●, wild-type barley; ○, chlorina-*f*2 mutant barley. (B) ●, wild-type wheat; ○, CD3 mutant wheat.

to ATP concentration quite differently than their wild-type counterparts. The thylakoid protein kinase activity of both mutants apparently saturated at lower ATP concentrations than the activities from the normal thylakoid membranes. The measured rates for protein kinase in the barley membranes at 0.8 mM ATP were 24 and 48 $pmol \cdot min^{-1} \cdot mg^{-1}$ of Chl for the wild-type and the chlorina-*f*2 mutant, respectively. Corresponding values for the wheat kinases measured at 0.75 mM ATP were 66 and 117 $pmol \cdot min^{-1} \cdot mg^{-1}$ of Chl for the wild-type and CD-3 mutant, respectively. Thus, all three Chl-*b*-deficient or lacking mutants had higher initial rates of thylakoid protein kinase activity on a Chl basis than the corresponding wild-type plants using endogenous protein substrates. The amount of protein (mg) present in these isolated thylakoid membranes per mg of Chl was as follows: wild-type sweetclover, 5.0; U374 mutant, 7.4; wild-type barley, 4.6; chlorina-*f*2 mutant, 9.1; wild-type wheat, 5.2; CD-3 mutant, 7.8. Thus, the amounts of thylakoid protein kinase activity are at least as high in the mutant relative to normal membranes when calculated on the basis of protein. The amount of protein per Chl

depends somewhat on the method used to isolate the membranes. Washing with EDTA lowers the protein present, but also appears to make the protein kinase activities less stable.

To test whether the differences in ATP concentration-dependence observed between the normal and mutant wheat or barley thylakoids was due to altered membrane surface potential (see Discussion), the initial rates of protein kinase activity in thylakoids from normal sweetclover were measured using two different Mg^{2+} concentrations (Fig. 6). The two Mg^{2+} concentrations used, 1 and 10 mM, will result in two different surface potentials for the thylakoid membranes. The plot of the ratio of kinase activity at high versus low Mg^{2+} concentration (Fig. 6A) indicates that the response to ATP concentration is the same under these two conditions. Similar data were obtained using tobacco (*Nicotiana tabacum*) thylakoid membranes in which the total thylakoid protein kinase activity is approx. 5-fold higher than in sweetclover, and the precision in the determination was correspondingly greater (data not shown). In this case, the ratio of activities at 1 and 10 mM Mg^{2+} versus ATP concentration was constant, but was increased to over 2.

The ability of mutant and wild type plants to demonstrate a state 1–state 2 transition was estimated from the 685 nm fluorescence emission of leaf tissue. The dark-adapted leaf material was initially excited with modulated broad-band blue light and the fluorescence emission allowed to reach a steady-state level. The tissue was then additionally exposed to unmodulated 710 nm light, which preferentially excites PS I [5]. Upon exposure of the leaf tissue to 710 nm irradiation there is an initial decline (Fig. 7) in the modulated 685 nm fluorescence which has been attributed to a reoxidation of plastoquinone [5], followed by a slow increase in fluorescence. The slow change in the 685 nm fluorescence is indicative of a decrease in excitation energy transfer from PS II to PS I and demonstrates the ability of the thylakoids in vivo to perform a State 1–State 2 transition. When the 710 nm irradiation is turned off there is a rapid increase in 685 nm fluorescence due to a

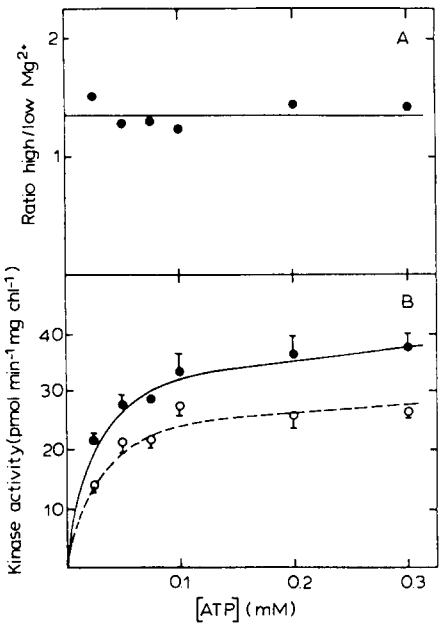

Fig. 6. Effect of 1 and 10 mM Mg^{2+} on the response of wild-type sweet clover thylakoid protein kinase activity to ATP concentration. Phosphorylation was measured using endogenous thylakoid proteins as substrates. (A) The ratio of the activity at 10 mM to the activity at 1 mM. (B) Activity measured in the presence of 1 (○) or 10 mM (●) Mg^{2+}.

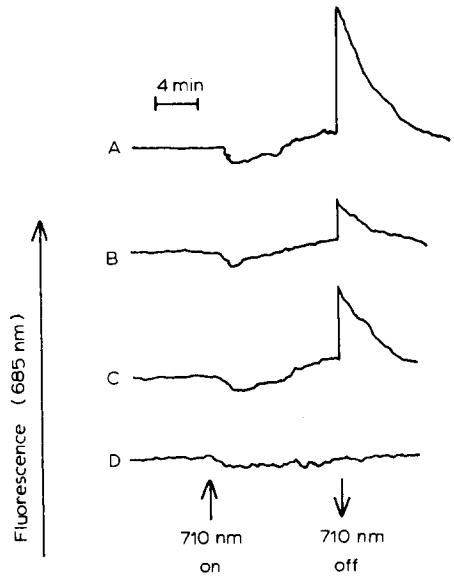

Fig. 7. The effect of 710 nm irradiation on 685 nm modulated fluorescence emission from leaves at room temperature. Symbols: (A) wild-type wheat; (B) CD-3 mutant wheat; (C) wild-type sweet clover; (D) U374 mutant of sweet clover. The magnitude of the 685 nm fluorescence prior to irradiation with 710 nm light is the same in all cases.

rapid photoreduction of PS II traps, followed by a slow decline as the leaf returns to state 2 in the absence of PS I light. Wild-type wheat (Fig. 7A) and sweet clover (Fig. 7C) were clearly able to perform the State 1–State 2 transition. The CD3 wheat mutant (Fig. 7B) also demonstrated a State 1–State 2 transition, although the extent of quantal redistribution, as indicated by the increase in 685 nm fluorescence emission in the presence of 710 nm irradiation, is less than in the wild-type wheat. In contrast, the U374 mutant of sweet clover (Fig. 7D) shows no slow enhancement of 685 nm fluorescence upon exposure to 710 nm irradiation, nor any rapid increase in fluorescence upon removal of 710 nm light. This suggests that the U374 mutant is unable to perform a State 1–State 2 transition. Adjustment of the relative intensities of the blue and red irradiations used in these experiments to compensate for the different absorption characteristics of the mutants could not induce a state transition in the U374 mutant.

Discussion

Most of the biochemical and biophysical studies on higher plant mutants deficient in Chl *b* have employed the chlorina-*f*2 mutant of barley. This report compares the chlorina-*f*2 and two additional mutants, sweet clover U374 and wheat CD3, with respect to their fluorescence emission spectra at 77 K, the response of their thylakoid protein kinase activities to ATP concentration and their ability to perform state transitions in vivo.

Fluorescence emission spectra of wild-type wheat and clover, containing a normal complement of chlorophyll *b*, exhibit a long-wavelength component with an emission maximum at approx. 740 nm. This fluorescence emission band is thought to at least partially arise from PS I chlorophyll molecules, and it is this long-wavelength (> 700 nm) emission that will be discussed further. Fluorescence emission spectra from leaves of CD3 wheat, partially lacking chlorophyll *b*, have an additional emission at 720 nm. Previously published emission spectra for thylakoids of the CD3 mutant at 77 K, while qualitatively similar, do not reveal such an intense emission at 740 nm [19]; these authors noted that the pigment-protein composition of this mutant changes with leaf age and this factor may be responsible for the difference. Fluorescence emission spectra of the U374 sweet clover mutant totally lacking chlorophyll *b* appear to exhibit only the 720 nm component; the 740 nm fluorescence emission maximum is not evident. This observation is similar to previously published spectra for the normal and chlorina-*f*2 barley [3].

These observations suggest that chlorophyll *b*-containing pigment-protein complexes are required in the thylakoid membranes to observe the 740 nm emission. It is probable that the 720 nm fluorescence emission is also present in the wild-type plants, but that it is obscured by the intense 740 nm emission. Kyle et al. [20] have shown that phosphorylation of thylakoid membranes at room temperature, which increases excitation energy transfer to PS I, enhances fluorescence emission at 720 nm. Our data are consistent with the hypothesis that fluorescence emission from score PS I chlorophyll molecules is primarily at 720 nm but that it is generally obscured by fluorescence emission at 740 nm from chlorophyll-*b*-containing complexes. Recent work with isolated PS I-enriched particles has suggested that a PS I-specific, chlorophyll-*b*-containing pigment-protein complex must be associated with PS I in order to observe a 740 nm emission and that its removal causes a shift in the emission maximum to approx. 720 nm [21,22]. More work with the mutants described herein will help to characterize further the origins of the long-wavelength fluorescence emission at 77 K in vivo.

Our data show that the U374 mutant totally lacking chlorophyll *b* is unable to exhibit a State 1–State 2 transition, and establish that this inability is not due to a loss of thylakoid protein kinase activity. This observation is consistent with data from the chlorina-*f*2 barley [4,5], suggesting that the ability of thylakoids to perform a State 1–State 2 transition appears to be dependent upon the presence of chlorophyll-*b*-containing complexes in the thylakoid membranes. Furthermore, leaves of the wheat CD3 mutant, which are partially deficient in chlorophyll *b*, perform a State 1–State 2 transition to a reduced extent compared with wild-type material. All three mutants exhibit thylakoid protein kinase activity. Phosphorylated thylakoid polypeptides have previously been fractionated by denaturing electrophoresis and visual-

ized by autoradiography for the cD-3 and normal wheat [19], for the chlorina-*f*2 and normal barley [23], as well as for the U374 and normal sweet clover (Markwell, J.P., unpublished results). In all cases, the normal and mutant plant thylakoid membranes have a number of thylakoid polypeptides which become phosphorylated upon incubation with ATP. Our data demonstrate that the mutants possess at least as much thylakoid protein kinase activity as their normal counterparts. This is not in agreement with the study by Haworth et al. [4] which reported much less activity in the chlorina-*f*2 mutant than in normal barley. We cannot explain this discrepancy, but note that we measure over 10-fold more activity (on a Chl basis) than they report in the normal barley thylakoids. This difference may be due to the different assays used.

Results of experiments in which the initial rates of thylakoid protein kinase activity were measured as a function of ATP concentration suggest a difference between the U374 mutant and CD3 or chlorina-*f*2 mutants. While the U374 mutant thylakoid protein kinase activity responds to ATP concentration in a manner similar to that of its corresponding wild-type, the thylakoid protein kinases of the Chl-*b*-lacking barley and Chl-*b*-deficient wheat mutants did not. There are a number of possible explanations for these differences and several will be examined. First, it is known that the thylakoid membrane contains multiple protein kinase activities [14,24] with differing K_m-values for ATP [14,25,26]. The protein kinase which phosphorylates the LHC-2 may have a higher K_m for ATP than the other kinases [25]. If this were the case, the absence of the LHC-2 from these mutant membranes could limit protein substrate availability. The observed situation in sweet clover would argue against this; these membranes are also lacking the LHC-2 [27] but phosphorylation is unaffected. Second, the absence of LHC-2, the most abundant component in the thylakoid membrane [28], could cause a decrease in the membrane surface charge density which in turn could cause an apparent lowering in the K_m for ATP. The effect of thylakoid membrane surface potential on the concentration of charged species near the thylakoid surface has been studied extensively by Barber and colleagues (cf. Ref. 29). This work has focused mainly on cations, although similar effects will also be observed for anions. Using the published pK_a values [30] for the conversion of ATP^{3-} to ATP^{4-} and for the chelation of Mg^{2+} with ATP, it can be assumed that $MgATP^{2-}$ will be the major form of this adenylate within the pH range of 6 to 8. Varying the Mg^{2+} concentration in the bulk phase from 1 to 10 mM will decrease the thylakoid surface potential and could increase the ATP concentration in the vicinity of membrane-solution interface. It has been observed previously [10,25] that increases in bulk Mg^{2+} concentrations of this magnitude markedly stimulate thylakoid protein kinase activity. The observations could be explained by a cation-induced change in surface potential mediating increased thylakoid protein kinase activity. However, when we tested this hypothesis by assaying thylakoid protein kinase activity at either 1 or 10 mM Mg^{2+} (Fig. 6), the apparent affinity for ATP was the same in both cases. This explanation for the differences in the thylakoid protein kinase activity in the barley and wheat mutants relative to their normal counterparts is also not supported by the experiments of Nakatani and Barber [31] who report that the normal and chlorina-*f*2 barley thylakoid membranes have identical surface charge densities.

As a final hypothesis, it is possible that the nature of mutation in the chlorina-*f*2 barley and CD-3 wheat may involve a loss or decrease in the high K_m thylakoid protein kinase. At the present time, we favor this hypothesis. It is known that developing tissues have much higher levels of thylakoid protein kinase activity than mature tissues [32], and it is possible that thylakoid protein kinase activity plays a key role in thylakoid membrane biogenesis and development. Loss of a thylakoid protein kinase activity could result in the inability to assemble or stabilize Chl-*b*-containing pigment-protein complexes.

In conclusion, the data presented in this report appear to indicate that Chl-*b*-deficient and lacking mutants can have altered thylakoid protein kinase activities. Whether these changes in enzyme activity are in some way responsible for the loss of Chl *b* and the Chl-*b*-binding apoproteins, or a result of it, is not known. There appears to be two different classes of Chl *b* mutants, however, based on their thylakoid protein kinase activities, and further work in this area is obviously required.

Acknowledgements

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