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IRON—SULFUR CENTERS OF THE CHLOROPLAST MEMBRANE

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A. INTRODUCTION

Photosynthesis in oxygen-evolving organisms involves the cooperation of two different photoreactions or photosystems. One, Photosystem II, generates in the light a strong oxidant, capable of oxidizing water to molecular oxygen, and concomitantly produces a weak reductant. The other photosystem, Photosystem I, produces in the light a weak oxidant and concomitantly generates a strong reductant which is capable of reducing pyridine nucleotide. It is widely accepted that electrons removed from water by Photosystem II

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are transferred to Photosystem I by a series of dark electron transfer reactions, some of which are coupled to the synthesis of ATP. This so-called noncyclic electron transfer sequence results in the production of stoichiometric amounts of O_2, reduced pyridine nucleotide in the form of NADPH and ATP. Photosystem I is also known to catalyze a cyclic electron transfer reaction which generates only ATP. Although complete agreement on the nature and identity of all the intermediates in these various reactions has not yet been reached, one hypothesis which describes these pathways is shown in Fig. 1. A more complete description of these electron transport pathways is beyond the scope of this review and the reader is referred to several recent works which provide a fuller coverage of many of the details (see refs. 1–3).

Numerous electron carriers in the electron transfer chain of oxygen-evolving systems have been identified and their proposed sites of function are shown in Fig. 1. These include *c*- and *b*-type cytochromes, quinones, a copper-containing protein and a flavoprotein. In 1962, an iron–sulfur protein of the ferredoxin type, chloroplast ferredoxin, was isolated from spinach leaves and found to function in photosynthetic NADP reduction [4]. This protein, which could be readily separated from bound components of the chloroplast membrane, was the only iron–sulfur protein believed to function in the photosynthetic electron transport chain. More recent studies, however, have led to the identification of several additional iron–sulfur centers which are bound to the chloroplast membrane and are involved in different electron transfer reactions. Because solubilization of these centers with their associated proteins has not yet been accomplished, little is known about their exact chemical nature, but in situ studies have given some indication of their function. In this article we will consider these iron–sulfur centers of the chloroplast membrane in some detail, emphasizing their functional roles. It is hoped this will

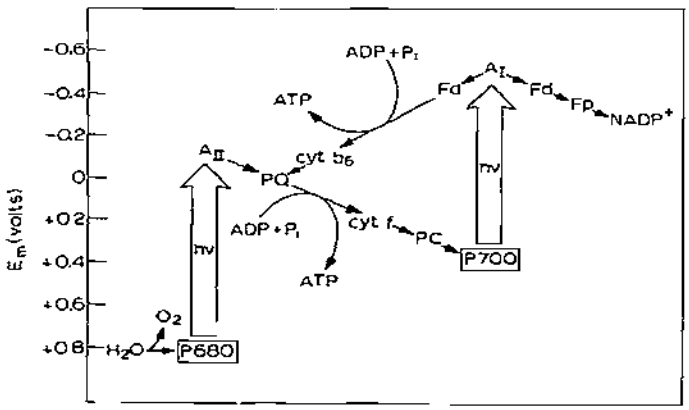

Fig. 1. A model for the electron transport pathway in chloroplast photosynthesis showing the cooperation of the two light reactions (Photosystems I and II) in noncyclic electron transfer from water to NADP.

serve as an introduction for readers not familiar with this class of electron carriers and their widespread distribution in photosynthetic tissues.

B. DETECTION OF IRON—SULFUR CENTERS IN CHLOROPLAST MEMBRANES

(i) Chemical analysis

Because iron—sulfur centers contain stoichiometric amounts of non-heme iron and "acid-labile" sulfur, it is possible to detect the presence of such centers on the basis of quantitative estimation of these cofactors by standard analytical techniques [5,6]. Analysis of chloroplast membrane fragments from which soluble chloroplast ferredoxin has been completely removed indicates the presence of a large amount of iron—sulfur centers (ca. 30 nmoles of iron and sulfur per mg chlorophyll [7,8]). This can be compared with the content of heme iron known to be associated with the other large class of electron carriers, the bound chloroplast cytochromes: approximately 5—10 nmoles of heme iron per mg chlorophyll can be estimated. Since the non-heme iron and sulfur are bound to the chloroplast membranes and not removed by treatments which release the chloroplast ferredoxin, these centers do not appear to originate from the soluble ferredoxin component.

Although chemical analyses of this type give a reliable indication of the presence of bound iron—sulfur centers, no information can be obtained from this data on the chemical nature of these centers or their functional roles. In order to characterize these centers in more detail, it is necessary to use spectroscopic methods which can unambiguously be applied to such types of molecules.

(ii) Optical spectroscopy

Studies of soluble iron—sulfur proteins of the ferredoxin type have been carried out for a number of years utilizing the optical properties of these proteins. In general, the ferredoxins are brownish or reddish-brown in color with major absorption bands in the visible spectral region, from 350 to 450 nm. Unfortunately, for the investigator attempting to work with photosynthetic systems, this spectral region is quite complex, due to contributions from chlorophyll and bound cytochromes. Thus, the Soret bands of these porphyrin-containing molecules have extinction coefficients of the order of 100 mM^{-1} cm^{-1} while the comparable extinction coefficients of most iron—sulfur centers are nearer to 10 mM^{-1} cm^{-1}.

Besides the problem of the intensity of the absorption bands, another serious problem is encountered with photosynthetic systems which makes optical detection of bound iron—sulfur centers difficult. Photosynthetic systems are known to contain a large amount of "antenna" chlorophyll, that is, chlorophyll which acts to trap light energy by absorption, but does not play a direct role in the conversion of this light energy into stable chemical pro-

ducts. The active redox carriers of the photosynthetic electron transport chain are present at a much lower level: approximately one of each carrier for every 400 chlorophyll molecules in a mature chloroplast. This large excess of bulk chlorophyll, although permitting efficient utilization of light energy, contributes a high spectral background against which the detection of the redox changes of the bound electron carriers is difficult to observe. Absorbance changes of cytochromes have been detected in situ by sensitive spectrophotometric techniques because of their large extinction coefficients but it has been impossible to detect redox changes of the bound iron—sulfur centers in intact chloroplasts because of this problem.

In enriched Photosystem I subchloroplast fragments, however, a spectral species, P430, has been detected by flash kinetic spectroscopy and the spectral changes are thought to be associated with membrane-bound iron—sulfur centers. The difference spectrum of P430 shows a broad band in the blue region, centered at approximately 430 nm, and no discernible absorbance changes beyond 500 nm. The difference spectrum of P430 resembles that of soluble ferredoxins in the ultraviolet region as well as the visible region, although the extinction coefficient appears to be greater than that of the soluble proteins. Because the properties of P430 have recently been extensively described by Ke [9], they will not be considered in any great detail in this presentation.

(iii) Electron paramagnetic resonance (EPR) spectroscopy

Reduced iron—sulfur centers display prominent EPR signals at temperatures below 80 K while no signals are present from the oxidized form of these molecules. These signals are caused by molecular antiferromagnetism between a high-spin iron(III) and a high-spin iron(II) which gives rise to an $S = \frac{1}{2}$ molecular paramagnetism with characteristic EPR g-values centered below 2.0, the so-called $g = 1.94$ type of EPR signal. This signal has a principal g-value in the range from 1.96 to 1.89, with $g = 1.94$ being the most commonly found principal g-value. Reduced iron—sulfur centers in photosynthetic systems display either rhombic ($g_x \neq g_y \neq g_z$) or axial ($g_\perp \neq g_\parallel$) EPR spectra. The rhombic EPR signal of reduced chloroplast ferredoxin is shown in Fig. 2 as being representative of the $g = 1.94$ type of signal and the three g-values are indicated. Characterization of iron—sulfur centers generally has been based on one of the two or three first-derivative resonance lines, usually the central $g = 1.94$ line because of less interference from other paramagnetic species in this g-value region. One major advantage of the EPR technique is that it affords a rapid, sensitive method for the unequivocal identification of an iron—sulfur center. In terms of application to photosynthetic systems, the EPR technique, being based on the magnetic properties of the sample, is not affected by the samples' optical characteristics, and the problems previously described for optical spectroscopy of iron—sulfur centers in photosynthetic systems become much less critical. EPR spectroscopy has another advantage over optical methods in

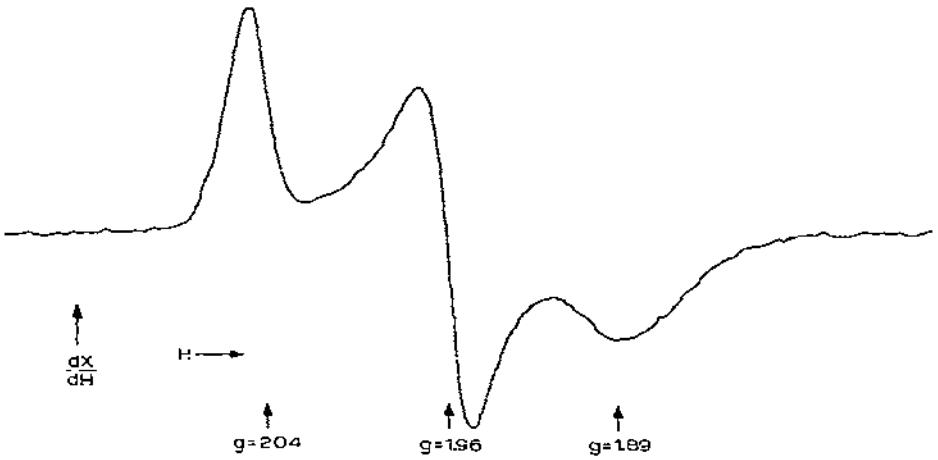

Fig. 2. The EPR spectrum of dithionite-reduced chloroplast ferredoxin from spinach recorded at 20 K.

that it can be used in a quantitative manner to determine the concentration of the paramagnetic center. For a more complete discussion of theoretical aspects of the EPR properties of iron—sulfur centers, see Orme-Johnson and Sands [10].

An important advance in the application of EPR spectroscopy to the study of membrane-bound electron carriers, including iron—sulfur centers, has been

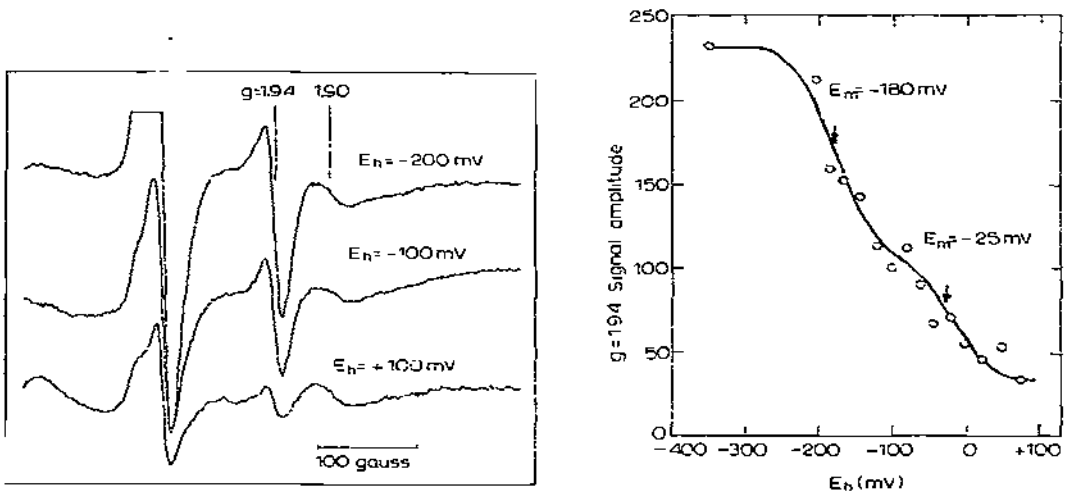

Fig. 3. EPR spectra of membrane-bound iron—sulfur centers in chromatophores from *Chlorobium* at defined redox potential. (From ref. 14.)

Fig. 4. Potentiometric titration of the $g = 1.94$ iron—sulfur centers in chromatophores from *Chlorobium*. (From ref. 14.)

the use of this technique in conjunction with redox potentiometry. Since most iron—sulfur centers have very similar g-values near $g = 1.94$, distinguishing between several centers in a particular system can be difficult. In a redox study, however, it is possible to distinguish multiple iron—sulfur centers on the basis of their midpoint oxidation—reduction potentials (E_m). The procedure involves the poising of the redox potential of the sample under investigation in the presence of suitable redox mediators to ensure equilibration between the carriers and the redox electrode (which is used to monitor the redox potential of the system), withdrawing samples at desired potentials, and examining their EPR signals at cryogenic temperatures where the signals of the iron—sulfur centers are detectable. It is generally possible to cover the redox span from +500 mV to —400 mV without complicated equipment. Detailed descriptions of these procedures have recently been published [11,12] and a theoretical consideration of the technique in relation to membrane-bound electron carriers is also available [13]. As an example of the application of this procedure to a photosynthetic system, a potentiometric titration of the iron—sulfur centers in a non-oxygen-evolving photosynthetic bacterium, *Chlorobium*, is shown in Fig. 3. Iron—sulfur centers with g-values of 1.90 and 1.94 are observed and, as shown in Fig. 4, it is possible to resolve the $g = 1.94$ signal into two different components, one with $E_m = -25$ mV and the second with $E_m = -175$ mV. Presumably these multiple centers have different functions in the electron transport processes of this organism [14].

C. LOW POTENTIAL IRON—SULFUR CENTERS

(i) A bound iron—sulfur center as the "stable" electron acceptor of chloroplast Photosystem I

The identification of bound iron—sulfur centers in chloroplasts was first made in 1971 on the basis of low temperature EPR analysis [8]. As shown in

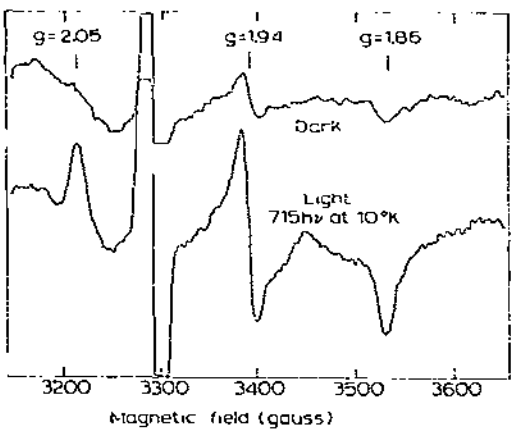

Fig. 5. Photoreduction with monochromatic light of wavelength 715 nm at 10 K of a bound iron—sulfur center in intact spinach chloroplasts.

Fig. 5, illumination of spinach chloroplasts at 10 K produces a paramagnetic species with EPR g-values (g_x = 1.86, g_y = 1.94 and g_z = 2.05) characteristic of the one electron reduction of an iron—sulfur center. Although the initial report on the detection of this center in chloroplasts utilized illumination at 77 K, subsequent technical improvements have allowed for illumination of samples directly in the EPR cavity at lower temperatures, such as that shown in this figure.

The g-values of the photoreduced iron—sulfur center are similar to, but not identical with, those of chloroplast ferredoxin (see Fig. 2), the only known iron—sulfur protein in chloroplasts at the time these measurements were made. The linewidths of the two sets of signals are, however, significantly different: the chloroplast ferredoxin's EPR linewidth is approximately 50 gauss while the new component has a linewidth of only 15 gauss. It was therefore suspected, and subsequentally proven, that the center observed in these chloroplasts did not originate from chloroplast ferredoxin. Since the latter protein can be removed from chloroplasts by washing of membrane fragments with dilute buffer, it was possible to show that its complete removal did not affect the EPR intensity of the new component. In addition, as will be discussed in detail below, the new iron—sulfur center was found in preparations of subchloroplast fragments which were totally devoid of chloroplast ferredoxin. Thus, the new center was first referred to as a "bound ferredoxin" to distinguish it from the "soluble" chloroplast ferredoxin, but because the exact chemical nature of this center has not yet been characterized in the isolated state, it is more appropriate to identify it in more general terms as a "bound iron—sulfur center" in order to avoid any confusion which might arise about the application of the term "ferredoxin".

As well as detecting the presence of this bound iron—sulfur center, this early work also revealed an important property of this center: it could be photoreduced at cryogenic temperatures. It is widely accepted, although not conclusively proven, that components in photosynthetic systems which undergo photoreactions at cryogenic temperatures are closely associated with primary photochemical events, that is, the events which are linked to the conversion of light energy into chemical products which occur at special sites known as the reaction centers (see refs. 1—3). The assumption is that normal diffusion-controlled chemical reactions do not occur at cryogenic temperatures while reactions linked to photon capture can still occur at an undiminished rate. On the basis of this reasoning the photoreduction of this iron—sulfur center would be related to a primary photochemical process in chloroplasts.

Further investigation has documented the association of the photoreducible iron—sulfur center with one of the two chloroplast photosystems, Photosystem I. Initial evidence [15] was obtained in studies in which chloroplasts were illuminated with monochromatic light. As shown in Fig. 5, far-red light of wavelength 715 nm, which is known to activate primarily Photosystem I, effectively reduces the bound iron—sulfur center. Red light, which activates both chloroplast photosystems, produces no further photoreduction after

far-red illumination. These findings directly relate the photoreducible iron—sulfur center with the Photosystem I reaction center.

Studies involving subchloroplast fragments enriched in either the Photosystem I or Photosystem II reaction center have confirmed the association of the bound iron—sulfur center with Photosystem I [15,16]. Various types of Photosystem I subchloroplast fragments have all shown the presence of the bound iron—sulfur center and it has been possible to demonstrate its photoreduction in these preparations [15—19]. Although quantitative EPR analyses of various Photosystem I enriched preparations have not been carried out in a systematic manner, on a qualitative basis it appears that as the reaction center becomes more enriched in such preparations (based on the content of the reaction center chlorophyll, P700), the enrichment of bound iron—sulfur centers shows a parallel increase. In contrast to this general pattern, one preparation, made with the detergent sodium dodecyl sulfate (SDS), shows unusual effects in terms of this iron—sulfur center, and these will be discussed in greater detail in a subsequent section.

A similar bound iron—sulfur center to that first observed in chloroplasts has also been found in a number of different preparations from oxygen-evolving algal species, including green algae [20], and from blue-green algae [20,21], as well as in enriched Photosystem I preparations from the latter [19]. In contrast, no bound iron—sulfur centers are observed in a highly enriched Photosystem II reaction center complex prepared from chloroplasts [22].

Because of the association of the bound iron—sulfur center with Photosystem I, numerous investigations of correlations between the iron—sulfur center and P700, the reaction center chlorophyll of this photosystem, have been described. The general object of this work has been to test the role of the photoreducible iron—sulfur center in the primary photochemistry of Photosystem I as a reaction center partner for P700. Many of these early studies, which will be discussed in detail, supported the assignment of the iron—sulfur center as this reaction partner and indicated this center functioned as the Photosystem I primary electron acceptor. More recent work, however, has led to a different conclusion and has suggested the possible existence of an intermediate electron carrier which may be photoreduced prior to the iron—sulfur center. Thus, the question of the true primary electron acceptor has become a matter of controversy, centering in some respects on definitions for the prerequisites for such a species. Before discussing this problem, it would be well to consider some of the findings on the role of the iron—sulfur center.

An important result which was consistent with a role for the iron—sulfur center in the Photosystem I primary photochemical event was the observed stoichiometry between photoreduced iron—sulfur center and photooxidized P700 [23,24]. Quantitative EPR estimation of the number of photoreduced iron—sulfur centers and photooxidized P700 molecules in unfractionated chloroplasts and a Photosystem I subchloroplast preparation showed a 1 : 1 correspondence after illumination and EPR analysis at 25 K. These two species were found to accumulate after illumination at this temperature and

hence the iron—sulfur center at least appears to be a relatively stable electron acceptor in Photosystem I in that some barrier exists for subsequent electron transfer events.

It is now well documented that under the conditions of the quantitative EPR study described above, the reaction between photooxidized P700 and photoreduced iron—sulfur center is irreversible. This reaction becomes reversible as the temperature is raised from 25 K to 77 K and above [25—27]. At temperatures from 77 K to 120 K, a back-reaction arising from a charge recombination between these two species has been demonstrated: there is a 40% decay of $P700^+$ in the dark at 120 K (after illumination at 25 K to produce the charge separation), and this corresponds to a 40% decay in the reduced iron—sulfur center in the same sample under similar conditions [25]. This result is consistent with a charge recombination between the reduced iron—sulfur center and the oxidized P700 in this temperature range. More extensive studies of this effect as a function of temperature have also been reported by two groups [26,27].

Additional evidence confirming the close association of the photoreducible

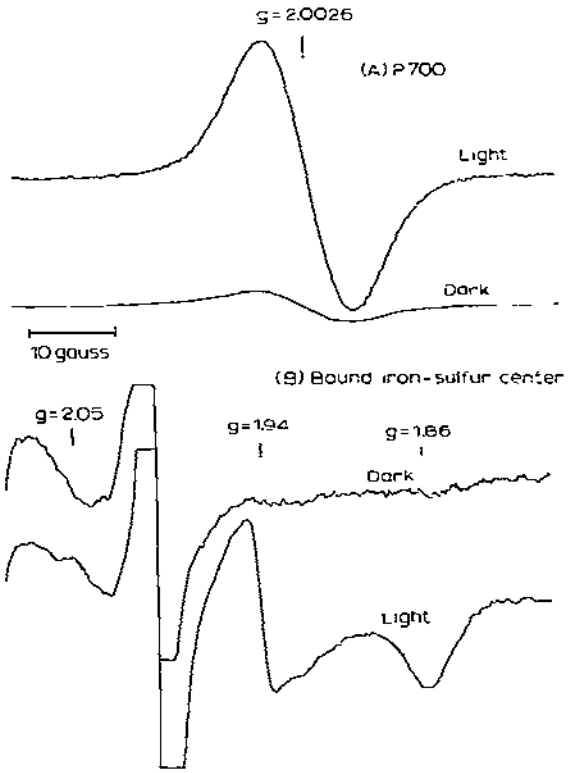

Fig. 6. Photochemical reactions at 15 K in a Triton Photosystem I reaction center complex from the blue-green alga, *Phormidium luridum*. (A) The photoreactions of P700; (B) the photoreactions of a bound iron—sulfur center. (From ref. 19.)

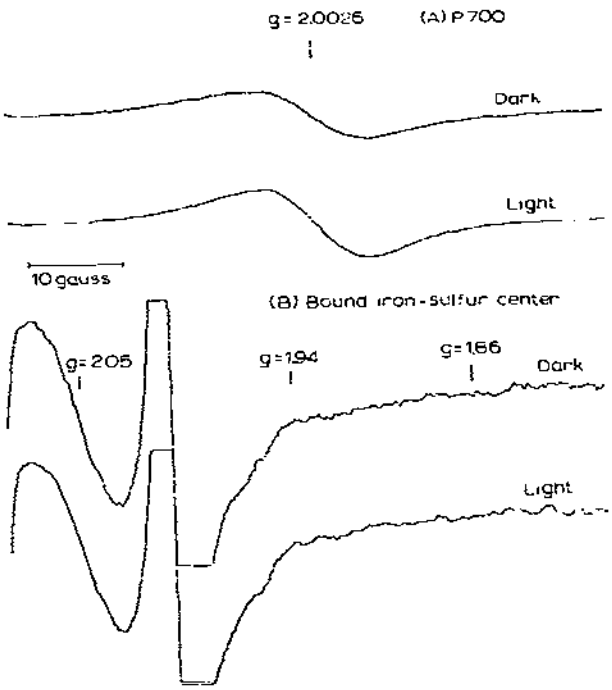

Fig. 7. Photochemical reactions at 15 K in an SDS Photosystem I reaction center complex from the blue-green alga, *Phormidium luridum*. (A) The photoreactions of P700; (B) the photoreactions of a bound iron—sulfur center. (From ref. 19.)

bound iron—sulfur center with P700 has come from recent work in which specific modification of the iron—sulfur center has been accomplished. It has been possible to use the detergent, SDS, to alter the iron—sulfur center in the Photosystem I reaction center from chloroplasts [28] and from the blue-green alga, *Phormidium luridum* [19]. In the latter case, a Photosystem I complex containing one P700 per 40 chlorophyll molecules when assayed at 300 K was prepared with the detergent Triton X-100 and a similar preparation was made with SDS. The Triton preparation displays photooxidation of P700, as indicated by the light-induced free radical signal at $g = 2.0026$, and also shows the photoreduction of the bound iron—sulfur center at 15 K, as shown in Fig. 6. The second complex, prepared from the same organism but with SDS instead of Triton X-100, has photochemical activity at 300 K in that P700 photooxidation still occurs, but when examined for low temperature photoactivity (Fig. 7), the preparation is inactive in that it shows no P700 photooxidation or iron—sulfur center photoreduction. Comparison of the two preparations after chemical reduction with a strong reductant which will reduce the iron—sulfur centers in the dark shows that the SDS preparation lacks these centers while they are present in the Triton preparation. Results of a similar nature have been obtained with a chloroplast Photosystem I pre-

paration which is subsequently treated with SDS [28]. These results indicate that in order to obtain a stable charge separation at cryogenic temperatures, the presence of a bound iron—sulfur center is required.

Another procedure which has been described which specifically modifies the iron—sulfur centers of Photosystem I is treatment with high concentrations of urea in the presence of ferricyanide [7,29,30]. This procedure apparently converts the acid-labile sulfur of the iron—sulfur centers to the S^0 state and results in loss of the ability of P700 to be photooxidized at 300 K. A good correlation has been found for the decrease in P700 photoactivity and the decrease in the content of acid-labile sulfur of a Triton X-100 Photosystem I preparation (one P700 per 25 chlorophyll molecules) during treatment with urea—ferricyanide. P700 does not appear to be altered by this treatment since detection from chemical oxidized minus reduced difference spectra revealed no decrease in the amount of this component. One significant advantage of the urea—ferricyanide treatment as compared with the SDS treatment is that the former is reversed by treatment of the fragments with dithiothreitol, i.e. acid-labile sulfur is restored and P700 photochemistry is regained. Although detailed physical studies utilizing EPR spectroscopy have not yet been done on the urea—ferricyanide treated material to determine the nature of the modification, this system seems to be particularly attractive for future investigations of the role of iron—sulfur centers in the Photosystem I reaction center complex.

The early evidence on the irreversibility of the Photosystem I charge separation at temperatures in the liquid helium range led to the proposal that the photoreducible iron—sulfur center functions in the primary electron acceptor complex of this photosystem [7,15,16]. The studies using SDS-modified Photosystem I preparations from chloroplasts and blue-green algae as well as those using urea—ferricyanide to modify the iron—sulfur centers, demonstrate that in order to obtain a relatively stable charge separation in the Photosystem I light reaction, the presence of a bound iron—sulfur center is required. What has emerged from this body of work is the concept that the electron lost from P700 during the photoact reaches the bound iron—sulfur center and that a barrier exists for the subsequent transfer of this electron to the next electron carrier. This is particularly evident from the studies of the charge separation at liquid helium temperature where photooxidized P700 and photoreduced iron—sulfur center are stable in the dark for long periods of time.

The term primary electron acceptor implies that the reduced species is the first reduced product formed during the charge separation event. Evidence to be discussed indicates that an intermediate electron carrier may be photoreduced prior to the bound iron—sulfur center. An analogous situation has been found in the reaction center from photosynthetic bacteria in which the component known as the primary electron acceptor (probably a non-heme iron—ubiquinone complex) is not the first reduced species formed but an another compound, possibly involving bacteriopheophytin, is reduced prior to the iron—ubiquinone complex [31]. It is clear from the studies which have

been discussed that the bound iron—sulfur center functions as a "stable" electron acceptor in the Photosystem I reaction center complex in a way apparently analogous to the iron—ubiquinone complex of the bacterial reaction center. This assignment does not preclude the identification of intermediates which might be formed prior to the reduced iron—sulfur center although it does suggest that an obligate, rapid electron transfer from such intermediates to the iron—sulfur center would have to occur in order to compete with wasteful back reactions.

(ii) A second low potential iron—sulfur center in the Photosystem I reaction center complex

Concomitant with the identification of the photoreducible iron—sulfur center, a second iron—sulfur center was also detected using EPR spectroscopy [7,16]. As shown in Fig. 8, reduction of Photosystem I fragments in the dark with a strong reductant (hydrogen gas plus hydrogenase or sodium dithionite in the presence of a catalytic amount of methyl viologen) produces an EPR spectrum similar to that observed after photoreduction at cryogenic temperatures but showing additional weaker resonance lines at g = 1.92 and 1.89. Longer incubations with reductant, also shown in this figure, produce a fully reduced spectrum which has g-values at 2.05, 1.94, 1.92 and 1.89. The latter spectrum is unusual in that it shows four g-values and the g-value at 1.86 has disappeared.

It was noted in an early report [7] that the fully reduced EPR spectrum characterized by four g-values cannot arise from a single, magnetically isolated iron—sulfur center since a maximum of three EPR g-values are obtained from

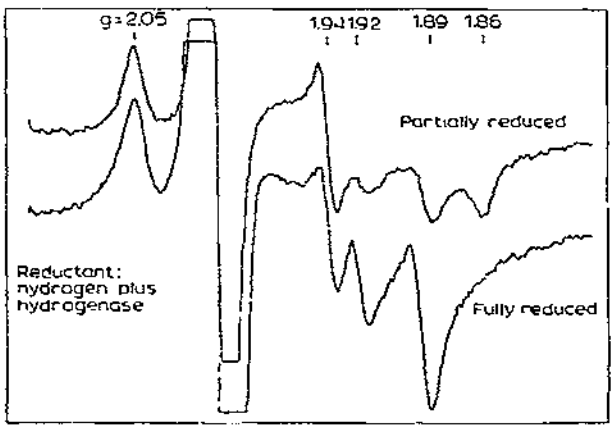

Fig. 8. EPR spectra of bound iron—sulfur centers in chloroplasts after dark reduction with hydrogen gas plus the enzyme hydrogenase in the presence of a catalytic amount of methyl viologen.

an $S = \frac{1}{2}$ transition metal ion, and it was proposed that such a spectrum could arise from the overlap of two different spectra, originating from two different iron—sulfur centers [7]. Subsequent work by other groups [32,33] has tended to confirm this interpretation of the chemically-reduced spectra. The two iron—sulfur centers are commonly referred to as A (g-values of 2.05, 1.94 and 1.86) and B (g-values of 2.05, 1.92 and 1.89) with Center A being the photoreducible component previously referred to.

The presence of two different iron—sulfur centers in Photosystem I has been confirmed by determination of the midpoint oxidation—reduction potentials of the centers. As can be noted from Fig. 8, Center A appears to be reduced prior to Center B in the chemical reduction, suggesting a more positive E_m. Ke et al. [32] and Evans et al. [33] have determined the E_m's of these centers and, although the two sets of values are not identical, they are similar: Center A has an E_m = —540 mV while the E_m of Center B is —590 mV. Although Ke et al. reported n-values of 2 for these centers, Evans et al. obtained results which were compatible with n = 1 titration curves for both centers, and the latter values seem more reasonable in terms of the known oxidation—reduction properties of other iron—sulfur centers. These redox potentials have been reported to be independent of pH in the pH range from 9 to 11.

One interesting feature which has emerged from these studies concerns the disappearance of the g = 1.86 signal over the course of the reductive titration. As noted in Fig. 8, this g-value is apparent in the spectrum of a partially reduced sample but is absent from the spectrum of a fully reduced sample. On the basis of qualitative examination of the intensity of the peaks in the fully reduced spectrum, it appears that the g = 1.86 signal undergoes a g-value shift to 1.89 although the other g-values of Center A remain unchanged. This conclusion has not been tested by careful simulation of these EPR spectra but it has been shown that a 1 : 1 relationship exists between Centers A and B [24] and that the g = 1.86 signal disappearance correlates well with the appearance of reduced Center B [32,33]. The possible significance of this g-value shift in relation to the nature of the iron—sulfur centers will be discussed in greater detail in a subsequent section.

Conflicting claims have been made as to the extent of photoreducibility of Center B at cryogenic temperatures. Ke et al. [32] have shown that little or no Center B photoreduction occurs in Photosystem I subchloroplast fragments poised at redox potentials where Center A is predominantly reduced. In contrast, Evans and Cammack [34] have shown some photoreduction of Center B in Photosystem I fragments poised at —560 mV prior to freezing and low temperature illumination. However, the extent of the photoreduction of this center seems small and does not approach the stoichiometric amount of Center A photoreduced.

We have observed a small but variable amount of photoreduction of Center B at cryogenic temperatures in various Photosystem I subchloroplast fragments but as in the above described case, this amount is approximately 10—25% of the amount of Center A photoreduced in the same preparation.

The significance of these observations in terms of the functioning of Center B in the photosystem I primary photochemistry is difficult to evaluate at this time, however, because of the variability of the reported results.

The presence of the two different iron—sulfur centers in the Photosystem I reaction center raises questions as to their chemical nature. Iron—sulfur centers in isolated proteins are known to contain either two or four iron atoms per cluster, and it is presumed that the bound centers also contain either two or four iron atoms. It has not yet been possible to isolate these centers in soluble proteins which retain their native properties so that this question cannot be answered on the basis of the characterization of an isolated protein.

A different approach employed by Cammack and Evans [35] utilizes reduction of the iron—sulfur centers in Photosystem I fragments after treatment with 80% DMSO and comparison of the EPR spectra of the treated material with those of similarly treated control proteins known to contain either two or four iron centers. The conclusion from these studies was that the fragments contained two four-iron centers and no two-iron centers were present. In the light of recent evidence that 80% DMSO treatment can facilitate dimer-to-tetramer conversion in synthetic analogs of iron—sulfur proteins [36], such a a conclusion may not be valid. Analysis of highly enriched Photosystem I fragments shows approximately ten atoms of non-heme iron and sulfur per P700 [8], a result which indicates the amount of iron is in excess of that required for the presence of two four-iron centers per reaction center. Further analysis of this type will require more purified reaction center preparations.

The relationship of the two iron—sulfur centers (Centers A and B) has also been a question of some interest. The close association of the centers is indicated by the *g*-value shift which occurs during reductive titration, but the meaning of this effect is not yet understood. Evans et al. [33] proposed that the disappearance of the $g = 1.86$ signal of Center A during reduction of Center B is due to changes in the shape of the Center A spectrum as a result of spin—spin interaction between the two reduced centers. Such a conclusion would necessitate that the two centers are extremely close, probably being located in the same protein molecule. It would be surprising that such a strong interaction only results in the shift of one *g*-value and not in more pronounced spectral perturbations. In addition, Ke et al. [18] have reported that it is possible, by Triton X-100 treatment of chloroplasts, to obtain a cytochrome complex which contains Center B but no Center A. The reduced EPR spectrum of this preparation shows a resonance line at $g = 1.94$ as well as at $g = 1.92$ and 1.89 so that the identity to the center in this preparation with either Center A or B is not clear. It has not yet been possible to obtain subchloroplast fragments which contain only one of the two centers, and this result, although of a negative nature, again confirms the close association of the two centers and suggests their possible presence in a single protein. Clearly, these questions await future clarification.

(iii) On the function of the bound iron—sulfur centers in the Photosystem I reaction center complex

Recent evidence has assigned a ubiquinone—iron complex as the primary electron acceptor in the reaction center of photosynthetic bacteria [31,37,38]. Electron transfer to this acceptor is independent of temperature and once formed, the primary reactants are relatively stable. The development of picosecond laser spectroscopy has allowed investigation of the bacterial primary reaction in a time domain unattainable until only recently. These studies [39—41] have demonstrated that a short-lived transient, possibly involving a reduced bacteriopheophytin molecule [42], is formed in less than 10 ps and donates an electron in 150 ps to the ubiquinone—iron complex. The latter carrier has usually been designated as the primary electron acceptor even though these experiments demonstrate that it is not the first reduced species formed during the charge separation. This designation is based on the relatively greater stability of the reduced form of the ubiquinone—iron complex compared with that of the reduced form of the transient; the latter has a lifetime of nanoseconds (based on measurements of a back reaction with $P870^+$ after extraction of the quinone) while the half-time for the back reaction between $P870^+$ and the reduced ubiquinone—iron complex is 30 msec [31,43]. The transient appears to function by transferring an electron to an acceptor which stabilizes the charge separation for a time long enough for chemical processes to be carried out. The existence of an intermediate in the charge separation event of photosynthetic bacteria has led to speculation about the existence of similar intermediates in the photochemical events of chloroplast photosynthesis. This will certainly be an area of active investigation in the future and the possible existence of such an intermediate in the case of Photosystem I has been suggested on the basis of recent optical and EPR experiments.

The earliest evidence that an electron acceptor preceded the bound iron—sulfur center came from experiments in which the photooxidation of P700 was measured at cryogenic temperatures under conditions where the bound iron—sulfur centers were in the reduced state [44]. Reduction of the iron—sulfur centers was brought about by freezing the sample during illumination: subsequent illumination at 6 K revealed a reversible P700 photooxidation with no change in the EPR signals of either of the two bound iron—sulfur centers. Subsequent work by Bolton [44—46] and Evans [45,47,48] and their co-workers led to the detection of a component "X" which showed a reversible EPR signal which correlated with that of P700 under the above described conditions. This component has EPR g-values of $g_x = 1.78$, $g_y = 1.88$ and $g_z = 2.08$; this spectrum is similar to that of a reduced iron—sulfur center (see ref. 49), although the major g-value is clearly at a lower field than is usually found. Bolton, on the other hand, has suggested a similarity of the signal with that from a quinone—iron complex [50]. The identity of the species responsible for this signal is open to further investigation.

The existence of an intermediate functioning prior to the iron—sulfur centers in Photosystem I has also been suggested on the basis of electrochemical studies of P700 photoreactions. Ke et al. [51] have reported that P700 photooxidation at cryogenic temperatures did not begin to become attenuated until a redox potential of −730 mV was applied to the sample. Since the bound iron—sulfur centers are fully reduced at this redox potential, an additional electron carrier must be functional under these conditions.

Independent evidence for the existence of a transient electron acceptor has more recently come from studies of flash-induced absorption changes in different Photosystem I subchloroplast fragments [52,53]. Kinetic components involved in a back reaction with $P700^+$ were detected at physiological temperature under different reducing conditions. Under mild reducing conditions, a decay time of 30 ms was observed and taken to be the rate of the recombination of $P700^+$ and reduced Center A. Under more strongly reducing conditions, two faster decay times of 250 μs and 3 μs could be observed and these were taken to indicate that two additional electron carriers function prior to iron—sulfur Center A. In a different Photosystem I preparation, isolated using the detergent SDS, a rapid decay of $P700^+$ was observed at 294 K as well as at 5 K. As has been previously discussed, preliminary analyses of these SDS Photosystem I preparations show the absence of bound iron—sulfur centers, but a transient charge separation has been demonstrated. Half times of decay of 10 μs at 294 K and 550 μsec at 5 K have been observed after laser flash activation of these preparations [53].

Studies with oriented chloroplast fragments have also been interpreted on the basis of the electron carrier X preceding the bound iron—sulfur centers as an electron acceptor [54]. The recent work is based on the observation of chemically induced, dynamic electron polarization (CIDEP) in the Photosystem I charge separation at room temperature [55] and has been interpreted by a radical pair mechanism [54]. To test the role of various proposed components in the acceptor complex, the orientation dependence of Center A, Center B, and X was considered. The iron—sulfur centers did not exhibit any orientation dependence, while the X^- species was found to undergo alignment after orientation. These results have been interpreted as indicating that X^- is the species responsible for the anisotropy of the polarized $P700^+$ spectrum and that X is the probable acceptor molecule in this radical pair model [54].

These optical and EPR experiments have given a consistent picture of at least one, and possibly two, electron acceptors preceding bound iron—sulfur Center A in the primary electron acceptor complex of Photosystem I. Thus, even though Center A can undergo photoreduction at cryogenic temperatures, this carrier appears to function as a secondary electron acceptor, and its role may be analogous to that of the ubiquinone—iron complex in the reaction center of photosynthetic bacteria. Several hypotheses describing the function of the two iron—sulfur centers have been put forward to describe the electron acceptor complex of Photosystem I, but, at this state of investigation, these can only be considered as tentative. Evans et al. [56] have proposed that a

linear sequence of carriers occurs, as shown in scheme 1

$$P700 \rightarrow \text{“X”} \rightarrow \text{Center B} \rightarrow \text{Center A} \rightarrow \text{Chloroplast ferredoxin} \quad (1)$$

According to this scheme, Centers A and B function in series and only Center A interacts with soluble chloroplast ferredoxin. This model takes into account the close association of the two centers but since the reactions of Center B at cryogenic temperature are not agreed upon, this formulation has not been proven. Bolton [50] has suggested a different function for the different iron—sulfur centers, as shown in scheme 2

$$P700 \rightarrow \text{“X”} \begin{cases} \rightarrow \text{Center B} \rightarrow \text{Cyclic electron transport} \\ \rightarrow \text{Center A} \rightarrow \text{Noncyclic electron transport} \end{cases} \quad (2)$$

According to this formulation Center A is involved in noncyclic electron transfer to NADP while Center B is involved in a cyclic electron transfer pathway around Photosystem I. This arrangement suggests that Centers A and B exist in discrete proteins which are probably located in different sites in the chloroplast membrane.

The differences in the above schemes deal mainly with the relationship of the two different iron—sulfur centers. A direct electron transfer from Center B to Center A has never been demonstrated and this is a critical matter in deciding between these alternatives. In addition, the recent optical experiments with SDS Photosystem I preparations suggest that additional electron acceptors might function before “X” although no information is yet available on the identity of such carriers. These studies are being actively pursued in several laboratories, and it is anticipated that the results of the next few years will provide some new insights on the components in the primary electron acceptor complex of Photosystem I.

D. THE “RIESKE” IRON—SULFUR CENTER

(i) EPR and redox properties

In 1975 Malkin and Aparicio identified a third chloroplast iron—sulfur center [57] which was characterized by the appearance of the following absorptions on reduction: $g_x = 1.78$, $g_y = 1.89$ and $g_z = 2.02$. Because of overlapping signals from other paramagnetic components in chloroplasts, this center has routinely been characterized on the basis of the g-value of 1.89; it has commonly been referred to as the “$g = 1.89$ iron—sulfur center”, and the EPR signal of the center is shown in Fig. 9. It should be noted that the linewidth of this signal is considerably broader than that of the low potential iron—sulfur centers (40 gauss versus 15 gauss), and thus it is more difficult to observe this signal in chloroplasts.

The EPR properties and the oxidation—reduction characteristics of the $g = 1.89$ iron—sulfur center are similar to those of a center first found by Rieske and co-workers in mitochondria and submitochondrial fragments [58,59] and subsequently found in a number of photosynthetic bacteria

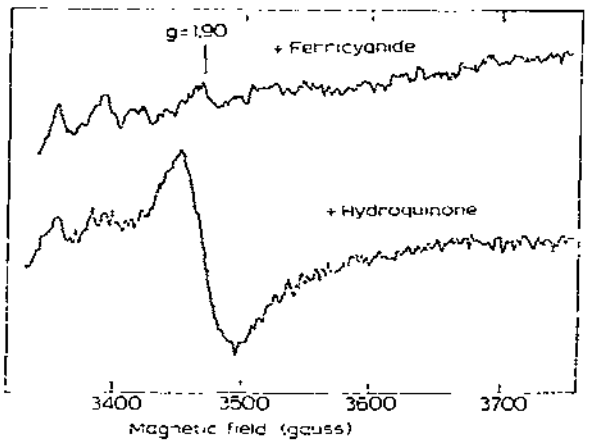

Fig. 9. EPR spectrum of the "Rieske" center in spinach chloroplasts after reduction with hydroquinone. (From ref. 57.)

[14,60—65]. Hence this type of center has also been referred to as the "Rieske" iron—sulfur center. Studies of substrate reduction in the presence and absence of inhibitors have shown that this center has a site of function near cytochrome c_1 in mitochondria. Consistent with this site is the presence of the "Rieske" center in mitochondrial Complex III (for a recent review, see ref. 66).

The "Rieske" center in chloroplasts is reducible by hydroquinone and therefore has a relatively high E_m. A value of +290 mV has been found for the center in unfractionated chloroplasts [57], and this is very similar to that of the mitochondrial center and to the centers which have been found in chromatophores from photosynthetic bacteria. The midpoint potential of the chloroplast center was found to be independent of pH from pH 6.0 to 8.0 [57] but, following the demonstration of a pK for this center in other systems [14,64], the dependence of potential on pH of the chloroplast center should be reinvestigated over a wider pH range.

(ii) Site of function in the chloroplast electron transfer chain

The site of function of the "Rieske" center in the chloroplast electron transfer chain was initially not known. Because of the reported E_m and its similarity to its mitochondrial counterpart, it might be expected to function in the region of the chloroplast *c*-type cytochrome, cytochrome *f*. A complex containing cytochromes b_6 and *f* has been isolated from spinach chloroplasts by Nelson and Neumann [67] and they have suggested this complex is analogous to Complex III and therefore might contain an iron—sulfur center, presumably the chloroplast "Rieske" center. We have been unable to detect this center in this complex after EPR analysis at cryogenic temperatures. Our prepara-

tions of the cytochrome complex, although containing non-heme iron, do not contain significant amounts of acid-labile sulfur. It appears that the non-heme iron may simply be a contaminant in the preparation and that the "Rieske" center is not a component of this isolated complex.

Two recent reports have presented some evidence that the "Rieske" center functions in the dark electron transfer chain connecting the Photosystem I and II light reactions [68,69]. It has been found that it is possible to reduce the "Rieske" center in the dark with the electron donor, duroquinol, and that this reduction is sensitive to an inhibitor, dibromothymoquinone (DBMIB), known to function at the plastoquinone site in the electron transfer chain. Another inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which functions between the primary electron acceptor of Photosystem II (A_{II}) and plastoquinone does not inhibit this reduction by duroquinol. Electron transfer from duroquinol through Photosystem I to the electron acceptor oxygen is also sensitive to DBMIB but not to DCMU. These results indicate duroquinol donates electrons on the oxidizing side of the Photosystem II primary electron acceptor, presumably through plastoquinone to reduce the "Rieske" center in a DBMIB-sensitive reaction.

In the dark, cytochrome *f* is also reduced by duroquinol in a DBMIB-sensitive reaction. Because the E_m of cytochrome *f* is considerably more positive than that of the "Rieske" center (estimates give values of approximately +360 mV for the E_m of cytochrome *f* in chloroplasts), one would predict from a thermodynamic basis that the "Rieske" center functions on the reducing side of cytochrome *f*, as shown in scheme 3

$$\begin{array}{c} \text{Duroquinol} \\ \downarrow \\ A_{II} \xrightarrow[\text{DCMU}]{} \text{Plastoquinone} \xrightarrow[\text{DBMIB}]{} \text{"Rieske" center} \rightarrow \text{cytochrome } f \end{array} \qquad (3)$$

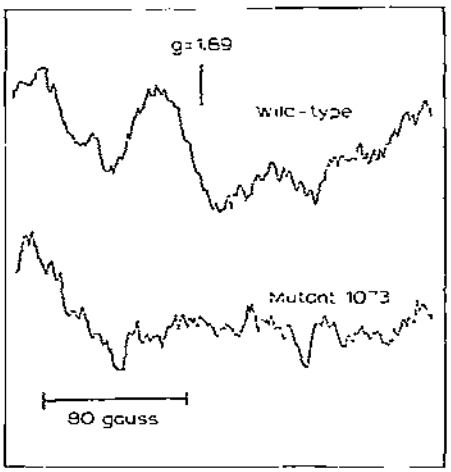

Fig. 10. The EPR spectrum of the "Rieske" center in wild-type and a mutant of *Lemna*. (From ref. 69.)

The localization of the "Rieske" center between plastoquinone and cytochrome *f* has been confirmed in studies with a mutant of *Lemna* (duckweed) [69]. Previous studies of partial electron transfer reactions in this mutant by Shahak et al. [70] indicated a block between plastoquinone and cytochrome *f*. Most notably, the chloroplasts from the mutant were able to photooxidize cytochrome *f* in a Photosystem I reaction but could not photoreduce the cytochrome even though electron transfer from water to the plastoquinone pool via Photosystem II was unaffected. As shown in Fig. 10, EPR examination of chloroplasts from wild-type *Lemna* show, on reduction, the $g = 1.89$ signal of the "Rieske" center while chloroplasts from the mutant have no $g = 1.89$ signal, indicating the mutant lacks a functional "Rieske" center. This analysis indicates the mutational block in the organism most likely involves the "Rieske" center and again is consistent with a site of function for this carrier between plastoquinone and cytochrome *f*.

E. CONCLUDING REMARKS

While early studies of iron—sulfur proteins resulted in the isolation and characterization of soluble ferredoxin type molecules from different sources and an understanding of the functions of these proteins in various cellular reactions, recent emphasis has been placed on the function of membrane-bound iron—sulfur centers in more complex organelles, such as the chloroplast and the mitochondrion (see refs. 71 and 72 for recent reviews on iron—sulfur centers of the mitochondrion). These results have all indicated the presence of multiple iron—sulfur centers, presumably functioning in a number of different electron transfer processes.

The close association of the low potential iron—sulfur centers with the Photosystem I reaction center has been well documented although the details of the function of these centers have not been fully resolved. The remaining iron iron—sulfur center, the "Rieske" center, appears to have a more defined role as an electron carrier in the dark electron transport chain and the recent demonstration that this carrier can function as a proton carrier under certain conditions as well as an electron carrier raises intriguing questions on a possible role for this carrier in energy-transduction processes according to a chemiosmotic type of mechanism (see ref. 73). Many problems remain to be solved and it is anticipated that the next few years will yield more definitive answers to some of these questions as well as giving a fuller understanding of the structure of these membrane-bound iron—sulfur centers.

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