Spatial Relationships between Specific Sites on Reconstituted Chloroplast Proton Adenosinetriphosphatase and the Phospholipid Vesicle Surface†

Richard A. Cerione,‡ Richard E. McCarty, and Gordon G. Hammes*

ABSTRACT: Two specific sulfhydryl groups on the γ subunit of the H^+–ATPase complex have been labeled with fluorescent maleimides prior to isolation of the complex from chloroplasts, and the distance between these sites on purified enzyme reconstituted into phospholipid vesicles and the surface of the bilayer has been determined by fluorescence resonance energy transfer. One of the sulfhydryl residues is freely accessible to maleimides (labeling can be performed in the dark), while the other can only be modified under conditions where the chloroplast membrane is energized by light. In the energy-transfer experiments, fluoresceinylmaleimide and pyrenylmaleimide served as the energy donors, while 5-(*N*-hexadecanoylamino)eosin, octadecylrhodamine and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine randomly distributed on the outer surface of the phospholipid vesicle bilayer served as energy acceptors. The results indicate that the distances of closest approach between the membrane surface and energy donor molecules attached to the sulfhydryl residues labeled in the dark and in the light are 44 and 42 Å, respectively. Energy-transfer measurements with pyrenylmaleimide at the site labeled in the dark and *N*-(1-anilino-4-naphthyl)maleimide at the site labeled in the light indicate that the maleimide groups attached to the two sulfhydryl residues are separated by 27 ± 6 Å. This is consistent with the different spectral properties of fluorescein at the two sites which suggest that fluorescein at the site labeled in the light is in a more hydrophobic region and is more rigidly bound. These findings indicate that at least a portion of the γ subunit extends quite far from the surface of the bilayer. Comparison with other results in the literature suggests a significant conformational change in the γ subunit occurs when thylakoids are energized by light.

The dicyclohexylcarbodiimide-sensitive H^+-ATPase (DSA)[1] complex in spinach chloroplasts catalyzes the terminal step of photophosphorylation, coupling ATP synthesis to the flow of protons down a proton gradient which is generated as a result of light-induced electron transport. This protein complex is composed of a soluble portion, coupling factor 1, CF_1, made up of five different polypeptides (α, β, γ, δ, and ϵ), and a membrane component, CF_0, which may contain as many as four types of polypeptides (Pick & Racker, 1979). The molecular weight of CF_1 has been reported to be 325 000 (Farron, 1970) and that of the complete DSA complex to be 405 000.[2] Nucleotide binding and catalysis occur on the α and β subunits of CF_1 (cf. Baird & Hammes, 1979), while a low molecular weight (M_r 8000) proteolipid in CF_0 appears to form the channel through which protons are pumped during ATP synthesis (Nelson et al., 1977). Coupling factor 1 can be stripped from the complete DSA complex by treatment with EDTA (Lien & Racker, 1971) and after isolation demonstrates a latent Ca^{2+}-dependent ATPase that can be activated by a variety of methods (Farron & Racker, 1970). The isolated DSA complex, which is activated by dithiothreitol prior to solubilization from the chloroplasts, shows optimal ATPase activity in the presence of Mg^{2+} (Pick & Racker, 1979). This complex also catalyzes P_i–ATP exchange (Winget et al., 1977; Pick & Racker, 1979) when reconstituted into phospholipid vesicles and ATP synthesis when reconstituted together with bacteriorhodopsin, a light-driven proton pump (Winget et al., 1977; Dewey & Hammes, 1981; Takabe & Hammes, 1981).

Electron micrographs of chloroplasts show that the CF_1 portion of DSA is spherical with a diameter of about 100 Å (Howell & Moudrianakis, 1967); however, little is known about the relationship of specific sites on CF_1 relative to sites on CF_0 or the membrane. Preliminary investigations of this problem were carried out by fluorescence resonance energy transfer measurements using labeled antibodies to CF_1 as energy donors and 5-(*N*-hexadecanoylamino)fluorescein as an energy acceptor (Baird et al., 1979). In the work reported here, two specific sulfhydryl groups on DSA, reconstituted into phospholipid vesicles, are labeled with fluorescent maleimides, while the surface of the vesicle is labeled with varying concentrations of different acceptor molecules. The donor sites are located on the γ subunit of CF_1 and are labeled prior to the isolation of DSA from the chloroplasts. Labeling at one of these sites is not dependent on the energy state of the membrane; i.e., labeling can occur in the light or dark, while the other site is only accessible to labeling in the light (McCarty & Fagan, 1973). Modification of the sulfhydryl exposed during illumination results in a partial inhibition of photophosphorylation which is sensitive to adenine nucleotides and uncoupling agents (McCarty & Fagan, 1973). The sulfhydryl group on the γ subunit exposed during illumination and that accessible in the dark are distinct. The γ subunit of CF_1 previously treated with dithiothreitol is remarkably sensitive to partial cleavage by trypsin (Moroney & McCarty, 1982a). Different fluorescent peptide fragments are generated by trypsin treatment of CF_1 modified with *N*-(1-anilino-4-naphthyl)maleimide at the light- or dark-accessible sulfhydryls, even though the pattern of trypsin digestion is identical.[3] In

† From the Department of Chemistry and Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received September 7, 1982.* This work was supported by grants from the National Institutes of Health (GM 13292) and the National Science Foundation (79-114746).

‡ National Institutes of Health Postdoctoral Fellow (GM 7160).

[1] Abbreviations: DSA, dicyclohexylcarbodiimide-sensitive H^+-ATPase; CF_1, coupling factor 1 of DSA; CF_0, coupling factor 0 of DSA; ANM, *N*-(1-anilino-4-naphthyl)maleimide; HAE, 5-(*N*-hexadecanoylamino)eosin; OR, octadecylrhodamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

[2] A molecular weight of 405 000 has been determined for sucrose gradient purified DSA with small-angle X-ray scattering methods (H. Paradies, U. Pick, and B. Baird, unpublished data).

[3] C. Nalin, R. Béliveau, and R. E. McCarty, unpublished observations.

0006-2960/83/0422-0769$01.50/0

light of the possible involvement of the γ subunit in proton translocation (cf. Weiss & McCarty, 1977), the location of these sites relative to the membrane surface is of interest.

Materials and Methods

Chemicals. Octyl glucoside was purchased from Calbiochem. Cholic acid, recrystallized prior to use (Kagawa & Racker, 1971), ATP (vanadium free), and Triton X-100 were purchased from Sigma Chemical Co. Asolectin (crude soybean phospholipids) was obtained from Associated Concentrates, Woodside, NY. [^{32}P]P_i was obtained from ICN and was heated to 90 °C in 2 N HCl for 2 h to hydrolyze any pyrophosphate which might be present. Cyanogen bromide activated Sepharose 4B was purchased from Pharmacia Fine Chemicals. Fluoresceinylmaleimide, pyrenylmaleimide, HAE, and OR were from Molecular Probes, Inc. *N*-(1-Anilino-4-naphthyl)maleimide was from Wako Pure Chemicals Industries, Ltd., Tokyo, Japan. Disodium fluorescein was obtained from Eastman Chemical Co., quinine sulfate from Aldrich Chemical Co., and NBD-PE from Avanti Biochemicals, Inc. All other chemicals were the best available commercial grades, and all solutions were prepared with deionized distilled water.

Preparation of Labeled Chloroplast Thylakoids and CF_1. Thylakoids were prepared from 500 or 1000 g of washed, deveined spinach by homogenization in 0.4 M sucrose, 0.01 M NaCl, and 0.02 M NaTricine (pH 8.0). All incubations with maleimides were carried out at room temperature in a medium that contained 50 mM NaTricine (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, maleimide as indicated, and thylakoids equivalent to 0.2 (for incubations in the dark) or 0.1 mg/mL (for incubations in the light). Accessible sulfhydryls were blocked by incubating thylakoids at 1–2 mg of chlorophyll/mL in the buffered salt medium with 2 mM *N*-ethylmaleimide for 5 min in the dark. Unreacted *N*-ethylmaleimide was removed by washing the thylakoids with the buffered sucrose solution. In one experiment, accessible groups were blocked with methyl methanethiosulfonate (50 μM). Incubation of the thylakoids with any of the sulfhydryl reagents in the dark at the concentrations tested had no effect on photophosphorylation.

Thylakoids with blocked accessible groups were used for the labeling of the group on the γ subunit of CF_1 that is exposed to reaction with maleimides only under energized conditions (McCarty & Fagan, 1973). The reaction medium contained 10 μM pyocyanine, a mediator of cyclic electron flow, in addition to the buffered salt solution. The incubations were carried out in 200-mL batches in a crystallizing dish (19 cm wide, 10 cm high). The medium was illuminated from the top and the bottom and was mixed with a magnetic stirrer. The light intensity at both surfaces was approximately 10^6 ergs/(cm^2·s). High light intensity is required since the incorporation of maleimides into CF_1 is critically dependent on the magnitude of the electrochemical proton gradient. After 2 min of illumination, dithiothreitol was added to 1 mM to remove unreacted maleimide. When the incubations with maleimides were completed, the thylakoids were collected by centrifugation for 5 min at 6000*g*. The thylakoids were resuspended in 10 mM NaCl to give a suspension of 0.1–0.2 mg of chlorophyll/mL and were collected by centrifugation at 9000*g* for 15 min. When CF_1 was prepared, the 10 mM NaCl wash was repeated twice. This procedure removes most of the ribulose-1,5-bisphosphate carboxylase. When DSA was to be prepared, the washed thylakoids were resuspended in a small volume of 0.2 M sucrose, 3 mM KCl, 3 mM $MgCl_2$, and 50 mM NaTricine (pH 8.0), collected by centrifugation for 15 min at 9000*g*, and resuspended in a minimal volume of the above medium.

The maleimides were used at concentrations that gave maximal inhibitions (60–80%) of photophosphorylation. Although the ϵ subunit of CF_1 in thylakoids contains a sulfhydryl that reacts with maleimides in the dark (McCarty & Fagan, 1973), the γ subunit can be selectively labeled when low maleimide concentrations and short incubation times are used. The γ subunit was the only component of either CF_1 or purified DSA that was labeled in either the light or the dark under the conditions selected. Fluoresceinylmaleimide was used at 40 μM for 2 min in the dark; pyrenylmaleimide was present at 25 μM and was incubated for 15 s in the dark. When CF_1 containing pyrenylmaleimide in the site accessible in the dark and ANM on the site exposed in the light was prepared, *N*-ethylmaleimide was added to 2 mM in the dark after the 15-s incubation of the thylakoids with 25 μM pyrenylmaleimide. This treatment prevents the reaction of ANM (20 μM) with any accessible groups that had not been blocked by the pyrenylmaleimide. In one experiment, accessible groups were blocked with methyl methanethiosulfonate and the thylakoids incubated in the presence or absence of ANM in the light. Dithiothreitol (10 mM) was added in the dark to remove the methane thiol blocking group. Methane thiol and dithiothreitol were removed by washing, and the control and ANM-treated thylakoids reacted with pyrenylmaleimide in the dark.

CF_1 was extracted by dilution of the 10 mM NaCl washed thylakoids to 50 μg of chlorophyll/mL with 0.75 mM EDTA (pH 8.0) at room temperature. The CF_1 was bound to DEAE-cellulose as described by Binder et al. (1978), except that 1 mL of settled DEAE-cellulose/mg of chlorophyll was used. The DEAE-cellulose was transferred to a column (room temperature) containing an equal volume of fresh DEAE-cellulose equilibrated with 20 mM sodium 2-(*N*-morpholino)ethanesulfonate (pH 6.0), 2 mM EDTA, and 1 mM ATP. The column was eluted with 3 column volumes each of this buffer, the buffer plus 0.1 M NaCl, the buffer plus 0.2 M NaCl, and, finally, the buffer plus 0.4 M NaCl. The CF_1 elutes in the 0.4 M NaCl column wash. The CF_1 was concentrated by ultrafiltration (Amicon PM-30 or XM-100 membranes) and precipitated with $(NH_4)_2SO_4$ (50% of saturation at 0 °C). When employed on a small scale (3–15 mg of chlorophyll), this procedure is convenient for the preparation of very highly purified CF_1.

Preparation of DSA. Partially purified DSA was prepared from market spinach by a modification of the procedure of Pick & Racker (1979; Cerione & Hammes, 1981); the concentration of cholate used to solubilize the complex from the chloroplasts was reduced to 0.1–0.2% (from 0.5%). The partially purified DSA was further purified by sucrose density centrifugation by a modification of the procedure described by Pick & Racker (1979). The partially purified enzyme was diluted to 7–9 mg/mL in 30 mM Tris–succinate (pH 6.5), 0.25% Triton X-100, 0.1 mM ATP, 0.1 mM EDTA, and 0.1–0.2% sodium cholate (final concentrations). This mixture (0.5 mL) was layered onto 4 mL of a 7–40% sucrose gradient with 0.1% or 0.2% sodium cholate, 30 mM Tris–succinate (pH 6.5), 0.1 mM ATP, and 0.1 mM EDTA. The centrifugation was performed in a Beckman SW-60 rotor for 5 h at 55 000 rpm at 2 °C. Fractions of 0.2 mL were collected at 4 °C from the top of the gradient tube. Both the partially purified and gradient purified enzyme preparations were stored in aliquots (0.05–0.2 mL) at −70 °C, following quick freezing in a dry ice–acetone slurry. Protein concentrations were determined according to Bensadoun & Weinstein (1976), using bovine

serum albumin as a standard. In calculating molar concentrations, a molecular weight for DSA of 405 000 was assumed.[2]

Partially purified and gradient-purified DSA were assayed for $[^{32}P]P_i$–ATP exchange activity according to Winget et al. (1977). The reconstitution of DSA, prior to assaying for exchange activity, was performed by the freeze–thaw procedure (Pick & Racker, 1979). The enzyme was diluted (final concentration of 0.5 mg/mL for partially purified DSA, 0.02–0.2 mg/mL for gradient purified DSA) in asolectin (40 mg/mL) which had been sonicated to clarity with a bath-type sonicator (Laboratory Supply Co. Inc., Hicksville, NY) in 80 mM NaTricine (pH 8.0) and 0.5 mM EDTA (Pick & Racker, 1979). Gradient-purified samples also were incubated in 50 mM dithiothreitol for 15 min (after reconstitution) which increases the activity (Pick & Racker, 1979). Specific activities for the partially purified enzyme were in the range 10–50 nmol of P_i/(min·mg) (37 °C). The specific activities after sucrose gradient purification are typically 3–5-fold higher, and sodium dodecyl sulfate gel electrophoresis patterns (Chua, 1980) for gradient-purified enzyme are similar to those obtained by Pick & Racker (1979).

Preparation of Sepharose 4B–Anti-Ribulosebisphosphate Carboxylase Column. The antiserum raised against ribulosebisphosphate carboxylase was a gift from Professor Andre Jagendorf. The thawed antiserum (1–2 mL) was added to an equal volume of 0.9% NaCl, and 2–4 mL of saturated ammonium sulfate was added. This suspension was allowed to stand at room temperature for 1 h and was centrifuged 15 min at 5000 rpm (room temperature). The pellet was taken up in 3 mL of 0.45% NaCl and was incubated 30 min with 3 mL of saturated ammonium sulfate. This supension was centrifuged 15 min at 10 000 rpm (room temperature), and the pellet was taken up in 0.5–1 mL of 10 mM sodium phosphate and 0.15 M NaCl and dialyzed 3 h against the same buffer (4 °C). The solution was then dialyzed overnight against 0.2 M sodium borate and 0.15 M NaCl, pH 8.3. The final protein concentration of the IgG solution was ~12 mg/mL.

One gram of cyanogen bromide activated Sepharose 4B was washed with 20 mL of 1 mM HCl several times by centrifugation (the supernatant being removed by aspiration) and was washed with the coupling buffer (0.2 M sodium borate, 0.15 M NaCl, pH 8.3). The dialyzed IgG (1.2 mL) and coupling buffer (2.4 mL) were added to the washed Sepharose and mixed for 2 h. The Sepharose 4B–IgG mixture was centrifuged 10 min at 10 000 rpm; the supernatant was removed, and 7 mL of 0.2 M sodium borate, pH 8.0, 0.15 M NaCl, and 1.0 M ethanolamine was added to the pelleted Sepharose 4B–IgG. This suspension was stirred for ~1 h and centrifuged, and the Sepharose 4B–IgG was washed 6 times alternatively with 0.4 M sodium acetate (pH 4.0), 0.5 M NaCl and the coupling buffer, and a final time with the coupling buffer. The affinity columns were prepared by adding the Sepharose 4B–IgG (0.8–2 mL) to 1-cm^3 (Pharmaseal) or 3-cm^3 (Becton-Dickinson) syringes partially plugged with glass wool. The gel was washed with approximately 10 volumes of the coupling buffer and then equilibrated in 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside prior to use.

Application of partially purified DSA preparations to these affinity columns (~1 mg/2 mL of Sepharose 4B) at 4 °C results in essentially complete removal of the contaminant ribulosebisphosphate carboxylase (which typically comprises 25–50% of the partially purified preparation) as visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The DSA complex is eluted in the void volume, with the removal of the contaminant protein being accompanied by a corresponding increase in specific P_i–ATP exchange activity. The columns can be reused after elution with approximately 5 volumes of 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside, 5–10 volumes of coupling buffer, 5–10 volumes of 3.5 M $MgCl_2$ and 50 mM Tris, and finally 5 volumes of the coupling buffer.

Preparation of DSA Samples for Energy-Transfer Measurements. The energy-transfer measurements between the sulfhydryl group on reconstituted DSA labeled in the dark with fluoresceinyl- or pyrenylmaleimide and the phospholipid vesicle surface labeled with OR or NBD-PE were performed as follows. The DSA was prepared from the labeled chloroplasts as described above. The two to three sucrose density gradient fractions with the highest $[^{32}P]P_i$–ATP exchange activity were first pooled from two gradients (~0.8–1.0 mL). These fractions were concentrated ~2-fold (room temperature) with 0.2 g of Sephadex G-25 (medium) (Cerione & Hammes, 1981) and then passed through a 3-mL centrifuge column (Penefsky, 1977) of Sephadex G-50 (fine) equilibrated in 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside.

The labeled DSA samples (0.25–0.5 mg in 0.5 mL) were applied to the Sepharose 4B–anti-ribulosebisphosphate carboxylase column (0.8 mL) at 4 °C and eluted at a rate of 1 mL/15–20 min with 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside to remove the labeled contaminant. The eluted DSA samples were concentrated, and reconstitution was performed by adding octyl glucoside (to a final concentration of 0.7%) to a 1:1 mixture of enzyme (0.2–1 mg/mL) and sonicated asolectin (40 mg/mL). This mixture was incubated on ice for 5 min and then an aliquot (15 μL) was diluted into 1 mL of 50 mM NaTricine (pH 8.0) and 1 mM EDTA for the energy-transfer measurements.

The energy-transfer measurements between the sulfhydryl group on DSA labeled in the dark with fluoresceinylmaleimide and the phospholipid vesicle surface labeled with HAE were performed with DSA which was not gradient purified in order to achieve a sufficient donor fluorescence signal over the background (acceptor) emission. The partially purified DSA preparation (2.5 mg/mL) was eluted through three consecutive 3-mL G-50 (fine) centrifuge columns, equilibrated with 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside, to remove fluorescent impurities. It was then eluted (0.7 mL) through the Sepharose 4B–anti-ribulosebisphosphate carboxylase column (4 °C) to remove labeled contaminant. The eluted DSA was concentrated with Sephadex G-25 (medium) and reconstituted by the octyl glucoside dilution procedure.

Partially purified DSA solubilized from chloroplasts that were labeled in the light with fluoresceinylmaleimide showed only a single fluorescent band corresponding to the position of the γ subunit after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. However, the partially purified preparations contained a great deal of pigment so gradient-purified samples were used in the energy-transfer experiments. The DSA gradient fractions from two to eight gradients containing the highest fluorescence were pooled, concentrated ~4-fold with Sephadex G-25 (medium), and eluted through a 3-mL G-50 fine centrifuge column equilibrated with 50 mM NaTricine (pH 8.0), 1 mM EDTA, and 10 mM octyl glucoside. The eluted DSA–fluoresceinylmaleimide samples were concentrated ~2-fold prior to reconstitution.

Vesicles containing NBD-PE, HAE, or OR were prepared by adding 0.01–0.4 mg of these compounds in 100 μL of dimethyl sulfoxide to 40 mg of asolectin in 1 mL of 80 mM

NaTricine (pH 8.0) and 0.5 mM EDTA. These solutions were sonicated to clarity in a bath sonicator ~3 min and then resonicated 15–20 s immediately before use. DSA–maleimide samples reconstituted into asolectin phospholipid vesicles containing 9% dimethyl sulfoxide were used as energy-transfer donors in the absence of acceptor. The DSA, reconstituted into phospholipid vesicles prepared in this manner, retain 80–100% of their P_i–ATP exchange activity.

The concentrations of HAE and NBD-PE were calculated by using the determined extinction coefficients of 1.19×10^5 M^{-1} cm^{-1} at 528 nm (HAE) and 2.24×10^4 M^{-1} cm^{-1} at 465 nm (NBD-PE). These values were determined from the absorption of HAE or NBD-PE in sonicated vesicles diluted into 50 mM NaTricine (pH 8.0) and 1 mM EDTA. Corrections were made for light scattering by measuring the absorption of an equal concentration of asolectin vesicles alone. The concentration of OR was calculated by using the determined extinction coefficient of 9.54×10^4 M^{-1} cm^{-1} at 564 nm. This value was obtained by comparing the absorption spectra for OR in vesicles with OR in acidic ethanol, using the extinction coefficient of 1.26×10^5 M^{-1} cm^{-1} for rhodamine in ethanol (Eastman Organic Chemicals, Catalog 50, 1979).

The stoichiometry of pyrenylmaleimide to DSA (or CF_1) was estimated by using an extinction coefficient for the pyrenylmaleimide adduct of 3.75×10^4 M^{-1} cm^{-1} at 343 nm (Holowka & Hammes, 1977); the stoichiometry of fluoresceinylmaleimide to DSA (or CF_1) was estimated from the absorbance at 495 nm by using an extinction coefficient of 7.08×10^4 M^{-1} cm^{-1} determined for fluoresceinylmaleimide in 50 mM NaTricine (pH 8.0) and 1 mM EDTA.

Preparation of CF_1 Samples for Energy-Transfer Measurements. Samples of CF_1 labeled in the dark with fluoresceinyl- or pyrenylmaleimide, and CF_1 labeled with fluoresceinyl- or pyrenylmaleimide in the dark and eosinmaleimide or ANM in the light, were eluted through a 3-mL centrifuge column of Sephadex G-50 (fine) equilibrated with 50 mM NaTricine (pH 8.0) and 1 mM EDTA. All CF_1 samples were then adjusted to the same protein concentration prior to performing the energy-transfer measurements. The absorption spectra for eosinmaleimide or ANM on modified CF_1 were obtained by correcting for the absorption of fluoresceinyl- or pyrenylmaleimide and for light scattering. The stoichiometry of eosinmaleimide to CF_1 was determined by assuming an extinction coefficient for eosin adducts of 8.3×10^4 M^{-1} cm^{-1} at 528 nm (Cherry et al., 1976); the stoichiometry of ANM to CF_1 was determined by using an extinction coefficient of 1.32×10^4 M^{-1} cm^{-1} at 355 nm (Kanaoka et al., 1973). The molar concentrations of CF_1 were determined by using an extinction coefficient of 0.483 mL/(mg·cm) (Bruist & Hammes, 1981) or by the method of Lowry et al. (1951), assuming a molecular weight of 325 000 (Farron, 1970).

Spectroscopic Measurements. Absorption spectra were obtained with a Cary 118 spectrophotometer. Steady-state fluorescence measurements were made with a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with corrected spectrum and polarization accessories. Fluorescence measurements were performed at room temperature (or with the fluorometer thermostated at 23 °C). The quantum yields for fluorescent-labeled CF_1 or DSA were determined by a comparative method (Parker & Rees, 1966) by using the relationship

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

where Q_1/Q_2 is the ratio of the quantum yields, F_1/F_2 is the ratio of the areas of the corrected emission spectra, and A_2/A_1 is the ratio of the absorbances at the exciting wavelengths of the two different fluorophores. Sodium fluorescein in 50 mM NaTricine (pH 8.0) and 1 mM EDTA and quinine sulfate in 0.1 N H_2SO_4 were used as standards and were assumed to have quantum yields of 0.92 (Weber & Teale, 1957) and 0.70 (Scott et al., 1970), respectively. The fluorescence emission of the fluoresceinylmaleimide and pyrenylmaleimide adducts of CF_1 were corrected for light scattering of the protein by subtracting the apparent emission for an equal concentration of CF_1 alone. In the case of labeled DSA, light-scattering corrections were made by subtracting the apparent emission for equal concentrations of unlabeled DSA reconstituted into vesicles. Steady-state polarization measurements, with corrections for the unequal transmission of horizontally and vertically polarized light by the emission monochromator grating, were made as previously described (Cerione & Hammes, 1982).

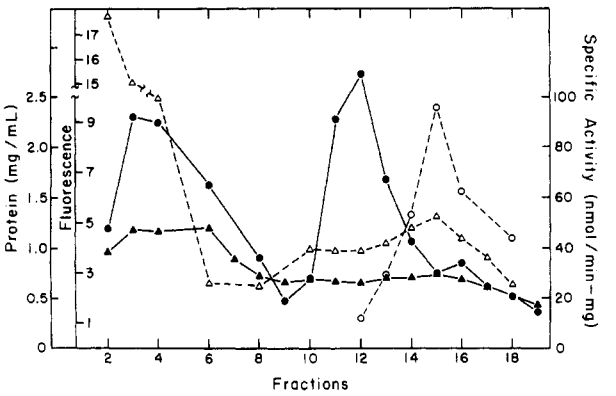

FIGURE 1: Purification of DSA and DSA–fluoresceinylmaleimide (labeled in the light) on sucrose density gradients (7–40%) at 4 °C. After separation, fractions from the gradient containing DSA were assayed for protein (●) and [^{32}P]P_i–ATP exchange activity (○); fractions from the gradient containing DSA–fluoresceinylmaleimide were assayed for protein (▲) and fluorescence [490 nm excitation, 513 nm emission (△)].

All energy-transfer measurements were performed at concentrations of acceptor which were sufficiently low (total absorbance at the excitation and emission wavelengths ≤0.05) so that inner-filter effects and trivial transfer were insignificant.

Results

The DSA complexes were labeled on the γ subunit with fluoresceinyl- or pyrenylmaleimide (~0.1–0.4 maleimide groups per mol of complex) prior to partial purification from the chloroplasts by detergent solubilization and ammonium sulfate fraction. These labeled DSA preparations were further purified by sucrose density gradient centrifugation. Typical gradient profiles for DSA isolated from untreated chloroplasts, and DSA first pretreated with *N*-ethylmaleimide, followed by treatment with fluoresceinylmaleimide in the light, are shown in Figure 1.

The [^{32}P]P_i–ATP exchange assays and sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicate that most of the DSA is located about 75–80% of the way into the gradient (fractions 14 and 15 consistently give the highest exchange activity and a peak in fluorescence). The early fractions (2–6) comprise protein associated with pigment, and the middle fractions (10–12) contain some DSA and the contaminant ribulosebisphosphate carboxylase. The amount of this contaminant was significantly reduced when the chloroplasts were washed in 10 mM NaCl prior to the detergent solubilization of DSA. This low salt wash was always performed when DSA–maleimide complexes were isolated. The DSA preparations labeled with fluoresceinyl- or pyrenylmaleimide in the dark show similar protein and activity

Table I: Energy-Transfer Parameters[a]

donor	location	emission maximum (nm)	Q_D	acceptor	absorption maximum (nm)	R_0 (Å)	L[b] (Å)
PM	D	375 395	0.30	NBD-PE	465	32.9	>30
FM	D	520	0.29	HAE	528	51.8	49[1] 44[2]
				OR	564	50.5	47[1] 44[2]
FM	L	523	0.54	OR	564	56.4	48[1] 42[2]

[a] D is the sulfhydryl site on DSA labeled in the dark, L is the sulfhydryl site labeled in the light, PM is pyrenylmaleimide, FM is fluoresceinylmaleimide, and all acceptors are located at the surface of the phospholipid vesicle. [b] The numbers designate the distance of closest approach between donor and acceptors, L, determined by least-squares analysis using eq 4 (1) or from comparison with Monte Carlo solutions to eq 2 (2).

profiles after gradient purification; in these cases some ribulosebisphosphate carboxylase was still present after detergent solubilization and was typically located in the middle fractions of the sucrose gradient (~1 mg/mL). Since the carboxylase is labeled with the fluorescent maleimides, to about the same extent as the γ subunit, elution through the Sepharose 4B–anti-ribulosebisphosphate carboxylase columns was necessary to remove any remaining contaminant, prior to making energy-transfer measurements.

The energy-transfer measurements between the fluorescent-labeled γ subunit on DSA reconstituted into phospholipid vesicles and the surface of the vesicle bilayer were performed with fluoresceinylmaleimide as the energy donor and HAE or OR as the energy acceptors. Since the diameter of CF_1 is approximately 100 Å (Howell & Moudrianakis, 1967) and the diameter of the vesicles is approximately 2000 Å (Racker et al., 1979), the results can be analyzed in terms of energy transfer between a specific energy donor on one plane and a uniform distribution of energy acceptors in a second plane. In this case, the ratio of the quantum yields in the presence and absence of the energy acceptor Q_{DA}/Q_D can be expressed as (Shaklai et al., 1977)

$$Q_{DA}/Q_D = (1/\tau_D)\int_0^\infty e^{-t/\tau_D}e^{-\sigma S(t)}\,dt \qquad (2)$$

with

$$S(t) = \int_L^\infty [1 - e^{-(t/\tau_D)(R_0/R)^6}]2\pi R\,dR$$

where τ_D is the fluorescence lifetime of the donor, σ is the surface density of energy acceptors, t is the time, R is the distance between the donor and an acceptor, L is the distance of closest approach between the donor and an acceptor, and

$$R_0 = 9.79 \times 10^3(K^2JQ_Dn^{-4})^{1/6} \qquad (3)$$

Here n is the refractive index of the medium, J is the spectral overlap integral, and K^2 is an orientation factor for dipolar coupling between the donor and acceptor. Calculation of Q_{DA}/Q_D requires numerical integration of eq 2; this ratio is a function of σ, R_0, and L so that determination of Q_{DA}/Q_D as a function of σ permits determination of the distance of closest approach, L. An empirical representation of the numerical integration for some specific cases has been presented by Wolber & Hudson (1979), and Monte Carlo calculations have been used to generate solutions (Snyder & Freire, 1982). An approximate series solution to the integration has been given by Dewey & Hammes (1980). The first term in the series, which is valid only for small extents of energy transfer, is

$$Q_D/Q_{DA} = 1 + (\pi\sigma R_0^2/2)(R_0/L)^4 \qquad (4)$$

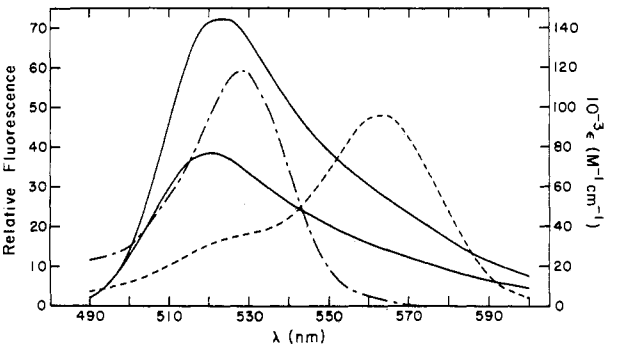

FIGURE 2: Spectral overlap of the corrected emission of CF_1–fluoresceinylmaleimide labeled in the light (upper solid line), and in the dark (lower solid line), with the extinction coefficient (ϵ) of HAE (– – –) and OR (– · –) in asolectin vesicles. The fluorescence excitation was at 480 nm. All spectra were taken in 50 mM NaTricine (pH 8.0) and 1 mM EDTA.

For the results presented, the density of acceptor molecules (number of acceptor molecules per Å^2 of phospholipid vesicle surface) was calculated by using the known molecular weights for the different acceptors and assuming a molecular weight of 740 for asolectin. The assumption was made that the acceptors are uniformly distributed on the outer and inner layers of the vesicles and that only acceptor molecules on the outer surface of the phospholipid bilayer contribute to the observed energy transfer. The values of R_0 were calculated with eq 3 and the measured spectral properties of the energy donors and acceptors. The overlap integrals were calculated from the spectra shown in Figure 2. The emission spectra for CF_1–fluoresceinylmaleimide (or pyrenylmaleimide) were used, rather than the spectra of the labeled DSA complexes since spectra of the former could be obtained more precisely. The wavelength of the emission maxima and the overall spectral shape of the excitation and emission are the same for the fluorescent-labeled CF_1 and DSA. The value of K^2 was assumed to be $^2/_3$, the dynamic average. Values of R_0 and some of the fluorescent properties of the various donor–acceptor combinations are presented in Table I.

The ratio of the quantum yields in the presence and absence of acceptors, Q_D/Q_{DA}, for DSA labeled with fluoresceinylmaleimide in the dark is shown in Figure 3 as a function of C, the product of the surface density of the acceptor and R_0^2. Similar extents of energy transfer are observed with HAE and OR as energy acceptors, which is consistent with the similar R_0 values for the two donor–acceptor pairs. The curves in Figure 3 represent plots for different values of L/R_0 obtained from the solution of eq 2 [as tabulated by Wolber & Hudson (1979)]. The experimental data fall within a range of 0.8–0.9 for L/R_0. More precise values for L/R_0 (0.87 and 0.84 for OR and HAE, respectively) were obtained by minimizing the

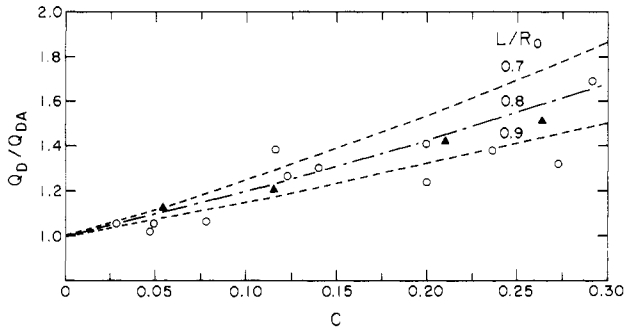

FIGURE 3: Ratio of the donor quantum yield in the absence and presence of acceptors [OR (○); HAE (△)], Q_D/Q_{DA}, vs. $C(\sigma R_0^2)$ for reconstituted DSA–fluoresceinylmaleimide labeled in the dark. The fluorescence measurements (480 nm excitation, 510 nm emission) were made in 50 mM NaTricine (pH 8.0) and 1 mM EDTA. The curves were calculated from eq 2.

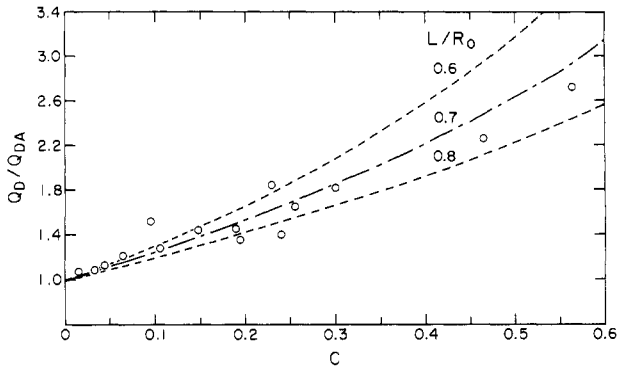

FIGURE 4: Ratio of the donor quantum yield in the absence and presence of acceptor (OR), Q_D/Q_{DA}, vs. $C(\sigma R_0^2)$ for reconstituted DSA–fluoresceinylmaleimide labeled in the light. Fluorescence measurement (480 nm excitation, 510 nm emission) were made in 50 mM NaTricine (pH 8.0) and 1 mM EDTA. The curves were calculated from eq 2.

root mean square deviation of the data from curves generated by Monte Carlo calculations (Snyder & Freire, 1982). The linear approximation (eq 4) gives $L/R_0 = 0.94$.

Pyrenylmaleimide and NBD-PE were used as an additional donor–acceptor pair in energy-transfer measurements. However, since R_0 is only about 30 Å, significant amounts of energy transfer were not observed.

Energy-transfer measurements between the site on γ labeled in the light and the surface of the vesicle bilayer were performed with fluoresceinylmaleimide as the energy donor and OR as the acceptor. The spectral properties for fluoresceinylmaleimide at the site labeled in the light show obvious differences from those for fluoresceinylmaleimide at the site labeled in the dark. The absorption and excitation maxima are shifted ~5 nm (from 495 at the dark site to 500 nm at the light site), and the quantum yield is increased nearly 2-fold. This latter increase is accompanied by a slight red shift in the emission maximum (from 520 to 523 nm) and by about a 2-fold increase in the fluorescence polarization (0.41 compared to 0.24). Figure 4 shows the plot of the ratio of the fluorescence of fluoresceinylmaleimide (at the site labeled in the light) in the absence and presence of OR, Q_D/Q_{DA}, vs. C. In this case, comparison with the solutions of eq 2 shown in Figure 4 indicates $L/R_0 \approx 0.7$, and the Monte Carlo fit of the data gives $L/R_0 = 0.74$. The linear approximation, eq 4, gives $L/R_0 = 0.85$. Accurate energy-transfer measurements between this same site on the γ subunit and the vesicle surface using fluoresceinylmaleimide–HAE as the donor–acceptor pair could not be made. The fluorescence from HAE interfered with the measurement of the donor fluorescence because the amount of donor present was very low (~0.1 fluoresceinylmaleimide/mol of DSA).

Table I summarizes the distances of closest approach between the labeled sulfhydryl sites on reconstituted DSA and the surface of the vesicle membrane.

The intramolecular distance between the two labeled sulfhydryl sites on the γ subunit was also examined; pyrenylmaleimide at the site labeled in the dark was used as an energy donor and ANM at the site labeled in the light as an energy acceptor. The value of R_0, 27.5 Å, for this donor–acceptor pair was calculated by using eq 3 and assuming $K^2 = {}^2/_3$. The efficiency of energy transfer (E) between these two sulfhydryl sites was determined by using the relationship

$$E = 1 - Q_{DA}/Q_D \quad (5)$$

and the distance, R, between these sites was calculated by using

$$R = R_0(E^{-1} - 1)^{1/6} \quad (6)$$

In experiments where labeling with pyrenylmaleimide in the dark (0.5/mol of CF_1) is performed first, followed by labeling with ANM in the light (0.3/mol of CF_1), the observed energy-transfer efficiency is 0.13. This corresponds to an efficiency of 0.4/mol of acceptor, if random labeling of donors and acceptors is assumed, resulting in a calculated distance between the two sites of 30 Å. However, some hindrance of the reaction of ANM with CF_1 might occur because of the pyrene bound at a nearby site; this would result in nonrandom labeling. For elimination of this possibility, all accessible sulfhydryl sites were first blocked (in the dark) with a CH_3–SH adduct. The ANM was then reacted with CF_1 in the light, followed by removal of the CH_3–SH with dithiothreitol and relabeling (in the dark) with pyrenylmaleimide. This does not, of course, eliminate the possibility of nonrandom labeling by pyrene. The stoichiometry of labeling was 0.15 ANM/mol of CF_1 and 0.5 pyrenylmaleimide/mol of CF_1. The energy-transfer efficiency found in this case is 0.65/mol of acceptor. This corresponds to a distance of 25 Å. The fluorescence polarizations for the donor and acceptor are 0.31 and 0.49, respectively. These yield upper and lower limits for K^2 of 0.10 and 3.2 (Dale et al., 1979) and thus a maximum uncertainty in the calculated distance of ±30%.

Fluoresceinylmaleimide (at the site labeled in the dark) and eosinmaleimide (at the site labeled in the light) also were used as a donor–acceptor pair. However, only a maximum distance between these sites (≤35 Å) could be determined by using this pair because of the low stoichiometry of the acceptor (<0.1/CF_1) and the large value of R_0 (~50 Å).

Discussion

The spatial relationships of two specific sites on the γ subunit of reconstituted DSA relative to the phospholipid vesicle surface have been examined by fluorescence resonance energy transfer. Fluorescent maleimides attached at two sulfhydryl groups on the γ subunit serve as the energy donors in these experiments, while eosin and rhodamine attached to long-chain carbon compounds and NBD conjugated to phosphatidylethanolamine serve as energy acceptors in the vesicles. The differential labeling of the sulfhydryl residues is dependent on the energy state of the chloroplast membrane (McCarty & Fagan, 1973); to ensure that only the γ sulfhydryl residues are labeled, the chloroplasts are exposed to low concentrations of the maleimide (μM) for only short periods of time (seconds). This results in typical stoichiometries of ≤0.4 mol of label/mol of DSA. The low stoichiometry of labeling, coupled with the small quantities of DSA obtained after purification (by sucrose gradient centrifugation and, in some cases, affinity chromatography), together with the ne-

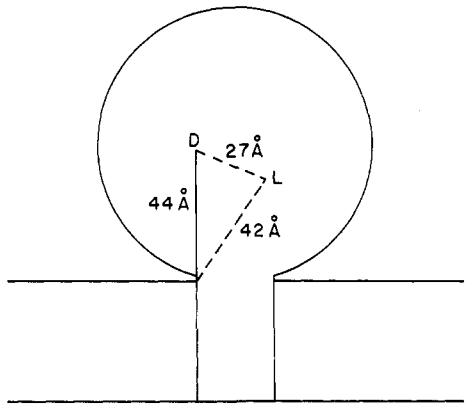

FIGURE 5: Schematic representation of the spatial relationships between the sulfhydryl group on the γ subunit labeled in the dark (D) and light (L) and the membrane surface. The circle represents CF_1. The approximate distances determined by energy-transfer measurements are indicated.

cessity of diluting the reconstituted DSA to eliminate trivial mechanisms of quenching resulting from high acceptor concentrations (inner-filter effects), is responsible for some scatter in the data. Still, good agreement is obtained in the energy-transfer efficiencies for two different acceptors (HAE and OR), and these results (L = 44 Å) are consistent with the observation of little energy transfer when a third donor-acceptor pair (pyrenylmaleimide–NBD-PE) with a much lower value for R_0 is used. In calculating the values for R_0, the assumption was made that $K^2 = {}^2/_3$ which represents the case where the donor and acceptor rotate rapidly relative to the fluorescence lifetime of the donor. The fluorescence polarizations for the fluoresceinylmaleimide and pyrenylmaleimide adducts at the sulfhydryl site labeled in the dark are 0.24 and 0.31, respectively. These would give a maximum uncertainty in the distance measurements of about ±25% (Dale et al., 1979) if the energy acceptor is assumed to have no rotational freedom. However, since the large number of acceptors in the vesicle bilayer is probably randomly oriented, the actual uncertainty in these calculations caused by the uncertainty in K^2 is likely to be less than ±10%. This is, in fact, suggested by the similarity in the calculated distances of closest approach using different acceptors. Since the eosin and rhodamine moieties of HAE and OR are charged at pH 8.0, these acceptor chromophores are very likely at the outer and inner surfaces of the vesicle bilayer. This is also true for the NBD moiety of NBD-PE which is conjugated at the phospholipid head-group region, thus ensuring that the measured distances of closest approach represent the distance to acceptors on the outer surface of the membrane. The thickness of the bilayer is at least 40 Å (Huang & Mason, 1978) which makes the contribution to energy transfer of acceptor molecules located at the inner surface of the bilayer ≤8%. Since the diameter of DSA is much smaller than the diameter of the octyl glucoside reconstituted DSA vesicles, the distances of closest approach between the donor sites and the bilayer surface were determined by assuming energy transfer occurs between a donor in one plane and a uniform distribution of acceptors in a second parallel plane. When the exact solution for energy transfer between two planes (Wolber & Hudson, 1979; Snyder & Freire, 1982) and the first term in the approximate series solution (Dewey & Hammes, 1980) are used, the differences in the distances of closest approach are consistent with the predicted deviations for the experimental range of σ and R_0/L (Dewey & Hammes, 1980). The linear approximation consistently overestimates L. The results suggest that the fluorescent maleimides at both sulfhydryl sites are ~40–45 Å from the closest acceptor molecule.

The spectral properties of fluoresceinylmaleimide at the two sulfhydryl sites on the γ subunit are quite distinct. The spectral shift and increased quantum yield for the site labeled in the light suggest it has a more hydrophobic environment; the increased polarization suggests rigid binding. The fluorescence of ANM bound to the site labeled in the light was previously found to be shifted about 14 nm to the blue compared to that bound to the site labeled in the dark (Béliveau et al., 1981). Since the site labeled in the dark requires very low reagent concentrations for rapid labeling and is labeled by a variety of reagents, it is probably readily accessible. The energy-transfer measurements indicate these two sites are 27 (±6) Å apart, which is consistent with the very different environments of the sites. (This distance is a weighted average of the two measurements, approximating the uncertainty for each measurement as ±30%.) On the other hand, the two sulfhydryl groups have been cross-linked by *o*-phenylenedimaleimide in the light, suggesting the two residues are ≤10 Å apart (Weiss & McCarty, 1977). Also other experiments suggest these two sulfhydryl groups can be oxidized to form a disulfide linkage (Andreo & Vallejos, 1976). The most likely explanation for the differences in the distance inferred from these experiments is that a conformational change can occur in the light which brings the sulfhydryl groups close together. Additional evidence for a change in the conformation of the γ subunit of CF_1 is that energization of thylakoids causes a dramatic increase in the sensitivity of the γ subunit to cleavage by trypsin to a 25-kilodalton fragment (Moroney & McCarty, 1982b). Of course, the fluorescent probes are large molecules, and if the two probes are pointed away from each other, the distance between the sulfhydryl groups could be 10–15 Å less than the measured distance between the transition moments of the probes. The results from the two different labeling procedures used suggest labeling at the two different sites is random when one of the sites is partially prelabeled.

The measured distances are summarized schematically in Figure 5. The distances of closest approach between the sites on DSA and the vesicle surface are not equal to the perpendicular distances to the surface because of the finite size of DSA. However, these measurements are consistent with previous results that suggest part of the γ subunit is quite distant from the membrane surface (Baird et al., 1979).

Acknowledgments

We are indebted to Dr. B. Snyder for carrying out the Monte Carlo calculations, to L. Lopresti for technical assistance, and to Kurt Warncke and Dr. Carlo Nalin for the preparation of the maleimide-labeled thylakoids and CF_1.

Registry No. ATPase, 9000-83-3.

References

Andreo, C. S., & Vallejos, R. H. (1976) *Biochim. Biophys. Acta 423*, 590–601.

Baird, B. A., & Hammes, G. G. (1979) *Biochim. Biophys. Acta 549*, 31–53.

Baird, B. A., Pick, U., & Hammes, G. G. (1979) *J. Biol. Chem. 254*, 3818–3825.

Béliveau, R., Phizicky, E., & McCarty, R. E. (1981) in *Energy Coupling in Photosynthesis* (Selman, B. R., & Selman-Reimer, S., Eds.) pp 179–184, Elsevier/North-Holland, New York.

Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.

Binder, A., Jagendorf, A. T., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298-6305.
Cerione, R. A., & Hammes, G. G. (1981) *Biochemistry 20*, 3359-3365.
Cerione, R. A., & Hammes, G. G. (1982) *Biochemistry 21*, 745-752.
Cherry, R. J., Cogoli, A., Oppliger, M., Schneider, G., & Semenza, G. (1976) *Biochemistry 15*, 3657-3661.
Chua, N.-H. (1980) *Methods Enzymol. 69*, 434-446.
Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161-194.
Dewey, T. G., & Hammes, G. G. (1980) *Biophys. J. 32*, 1023-1035.
Dewey, T. G., & Hammes, G. G. (1981) *J. Biol. Chem. 256*, 8941-8946.
Farron, F. (1970) *Biochemistry 9*, 3823-3828.
Farron, F., & Racker, E. (1970) *Biochemistry 9*, 3829-3836.
Holowka, D. A., & Hammes, G. G. (1977) *Biochemistry 16*, 5538-5545.
Howell, S. H., & Moudrianakis, E. N. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1261-1268.
Huang, C., & Mason, J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308-310.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477-5487.
Kanaoka, Y., Machida, D., Machida, M., & Sekine, T. (1973) *Biochim. Biophys. Acta 317*, 563-568.
Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 547.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McCarty, R. E., & Fagan, J. (1973) *Biochemistry 12*, 1503-1507.
Moroney, J. V., & McCarty, R. E. (1982a) *J. Biol. Chem. 257*, 5910-5914.
Moroney, J. V., & McCarty, R. E. (1982b) *J. Biol. Chem. 257*, 5915-5920.
Nelson, N., Eytan, E., Notsani, B. E., Sigrist, H., Sigrist-Nelson, K., & Gitler, C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2375-2378.
Parker, C. A., & Reese, W. T. (1966) *Analyst (London) 85*, 587-600.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Pick, U., & Racker, E. (1979) *J. Biol. Chem. 254*, 2793-2799.
Racker, E., Violand, B., O'Neal, S., Alfonzo, M., & Telford, J. (1979) *Arch. Biochem. Biophys. 198*, 470-477.
Scott, T. G., Spencer, R. D., Leonard, M. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687-695.
Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977) *Biochemistry 16*, 5585-5597.
Snyder, B., & Freire, E. (1982) *Biophys. J. 40*, 137-146.
Takabe, T., & Hammes, G. G. (1981) *Biochemistry 20*, 6859-6864.
Weber, G., & Teale, F. W. J. (1957) *Trans. Faraday Soc. 53*, 646-655.
Weiss, M. A., & McCarty, R. E. (1977) *J. Biol. Chem. 252*, 8007-8012.
Winget, G. G., Kanner, N., & Racker, E. (1977) *Biochim. Biophys. Acta 460*, 491-499.
Wolber, P. K., & Hudson, B. S. (1979) *Biophys. J. 28*, 197-210.

Inhibition of the Dehydrogenase Activity of Sheep Liver Cytoplasmic Aldehyde Dehydrogenase by Magnesium Ions†

Adrian F. Bennett, Paul D. Buckley, and Leonard F. Blackwell*

ABSTRACT: Magnesium chloride caused inhibition of the dehydrogenase activity of sheep liver cytoplasmic aldehyde dehydrogenase at all concentrations between pH 6 and 8 with no increase in the number of functioning subunits. There was also no decrease in the molecular weight as determined by gel filtration and laser light scattering experiments, results which are markedly different from those reported for the horse liver mitochondrial aldehyde dehydrogenase. There were changes in the spectroscopic and fluorescence properties of the enzyme, and enzyme-bound NADH, in the presence of magnesium ions. Steady-state inhibition studies revealed that magnesium ions exerted their inhibitory effect by decreasing V_{max} for the reaction by binding to a metal ion binding site which was distinct from the coenzyme and substrate binding sites. The biphasic nature of the Lineweaver-Burk plots at high (millimolar) concentrations of propionaldehyde was shown to be consistent with a steady-state model in which two binding sites (a catalytic low-K_m binding site and a high-K_m modifier binding site) for propionaldehyde exist. Pre-steady-state kinetic studies showed that $MgCl_2$ had no effect on the rates of NAD^+ or NADH binding or on the rate constants for the bursts in production of NADH or proton release. However, the dissociation constants for E·NAD^+ and E·NADH were significantly decreased in the presence of $MgCl_2$, and the rate constants for dissociation of the coenzymes were shown to be decreased. At high concentrations of propionaldehyde, the inhibitory effect of $MgCl_2$ could be almost entirely attributed to the tighter binding of NADH, but at low propionaldehyde concentrations, and for aromatic aldehydes, a more complex mechanism of inhibition must exist since the magnitude of the reduced k_{cat} values was almost an order of magnitude less than the reduced value of the decay constant for the slow step of the NADH displacement process.

Although aldehyde dehydrogenase (EC 1.2.1.3) are not metalloenzymes, a number exhibit a marked sensitivity to the presence of some metal ions. For example, Stoppani & Millstein (1959) found that yeast aldehyde dehydrogenase is inhibited by a wide range of metal ions, and Venteicher et al. (1977) showed that a variety of divalent and trivalent metal ions (such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, La^{3+}, and Gd^{3+}) affect the F_1 and F_2 isoenzymes of aldehyde dehydrogenase

† From the Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand. *Received August 20, 1982.*

0006-2960/83/0422-0776$01.50/0