Proton–Adenosinetriphosphatase Complex of Rat Liver Mitochondria: Effect of Its Inhibitory Peptide on Adenosine 5′-Triphosphate Hydrolytic and Functional Activities of the Enzyme†

Nitza M. Cintrón,‡ Joanne Hullihen, Klaus Schwerzmann, and Peter L. Pedersen*

ABSTRACT: The adenosinetriphosphatase (ATPase) inhibitor peptide of rat liver mitochondria [Cintrón, N. M., & Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 3439–3443] has been examined in detail with respect to its effect on the adenosine 5′-triphosphate (ATP) hydrolytic and functional activities catalyzed by the H^+–ATPase of inverted inner membrane vesicles from the same source. The peptide inhibitor maximally inhibits the ATPase activity of the H^+–ATPase when enzyme and inhibitor are incubated near pH 7.0 for ≥3 min prior to assay in the presence of the substrates ATP and Mg^{2+}. Inosine 5′-triphosphate (ITP) and guanosine 5′-triphosphate (GTP), which are also effective substrates for the enzyme, will substitute for ATP in the preliminary incubation. When the ATP hydrolytic activity taking place during the preliminary incubation is prevented by addition of ethylenediaminetetraacetic acid (EDTA), the peptide inhibitor is rendered ineffective as an inhibitor of the H^+–ATPase. Adenosine 5′-diphosphate (ADP) and P_i either separately or together are unable to replace ATP in the preliminary incubation. It is suggested that an intermediate H^+–ATPase complex containing bound MgATP, ADP, and/or P_i interacts with the inhibitor peptide. Kinetic experiments show that in tris(hydroxymethyl)aminomethane (Tris)–HCO_3 buffer velocity vs. [ATP] response patterns are hyperbolic (i.e., typical Michaelis–Menten) when no inhibitor is present, whereas when the ATPase inhibitor peptide is present, such patterns are biphasic. The inhibitor appears to unmask at least one ATP site on the rat liver H^+–ATPase that is functionally silent in Tris–HCO_3 buffer. Under conditions where the ATPase inhibitor peptide maximally inhibits ATPase activity of the H^+–ATPase of inverted inner membrane vesicles, it maximally inhibits also the ATP-dependent NADH–$NADP^+$ transhydrogenase and the ATP-dependent succinate-linked NAD^+ reduction activities supported by the enzyme. Under these conditions, the ATP–P_i exchange reaction catalyzed by the enzyme is inhibited about 50%, and the ATP synthetic activity is unaffected. The ATP–P_i exchange reaction can be further inhibited (≥75%) by addition of higher concentrations of inhibitor peptide. Results reported here describe an unusual relationship between an enzyme inhibitor and its target enzyme in that the catalytic activity of the enzyme itself is required to "prime" formation of a nonproductive inhibitor–enzyme complex. They also supply new information about the mode of interaction between the inhibitor protein and the H^+–ATPase. Finally, they show that the inhibitor peptide of rat liver is primarily a unidirectional inhibitor, which suppresses ATP-dependent processes while exerting little effect on the steady-state rate of ATP synthesis.

Protein inhibitors of H^+–ATPases[1] have been isolated from bovine heart (Pullman & Monroy, 1963; Horstman & Racker, 1970; Brooks & Senior, 1971), rat liver (Chan & Barbour, 1976; Cintrón & Pedersen, 1979), yeast (Satre et al., 1975; Ebner & Maier, 1977; Satre et al., 1979), chloroplasts (Nelson et al., 1972), and bacteria (Smith et al., 1975; Smith & Sternweis, 1977). An ATPase inhibitor protein has been isolated also from myofibrils (Greaser & Gergely, 1971; Wilkinson et al., 1972; Burtnick et al., 1975). These inhibitors are small peptides of less than 15 000 molecular weight that are highly resistant to extremes of pH, temperature, and ionic strength. They are also highly specific in their mode of action, inhibiting only certain enzymes catalyzing ATP (or NTP) hydrolytic reactions but not phosphotransferases or enzymes catalyzing other hydrolytic reactions (Pullman & Monroy, 1963; Horstman & Racker, 1970; Brooks & Senior, 1971; Cintrón & Pedersen, 1979).

A previous report from this laboratory summarized conditions for the purification of a homogeneous ATPase inhibitor from rat liver mitochondria (Cintrón & Pedersen, 1979). The rat liver inhibitor was shown to be about 2500 daltons larger than the bovine heart inhibitor of Pullman & Monroy (1963) and about 5000 daltons larger than ATPase inhibitors of yeast (Satre et al., 1975; Ebner & Maier, 1977; Satre et al., 1979). It was found also to contain significantly more lysine residues than the bovine heart and yeast inhibitors (Cintrón & Pedersen, 1979).

Experiments described in this paper were carried out with the purpose of better understanding the interaction of the rat liver ATPase inhibitor peptide with the H^+–ATPase of inverted inner mitochondrial membrane vesicles from the same source. Specifically, experiments were carried out with three objectives in mind. First, it was essential to establish optimal conditions for interaction of the rat liver inhibitor with the rat liver H^+–ATPase. A critical unanswered question along these lines is whether or not the ATPase activity, which takes place in the prior incubation medium, is essential to support formation of an inactive H^+–ATPase–inhibitor complex. Second, since it is well established that bicarbonate is an anion activator of rat liver mitochondrial H^+–ATPase (Lambeth & Lardy, 1971; Mitchell & Moyle, 1971), it was of interest to examine the

† From the Laboratory for Molecular and Cellular Bioenergetics, Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received August 10, 1981.* This work was supported by U.S. Public Health Service Grant CA 10951 from the National Institutes of Health, by National Institutes of Health Training Grant GM 00184, and by Fogarty Fellowship 1-F05-TWO 2755.

‡ Present address: National Aeronautics and Space Administration, Lyndon B. Johnson Space Center, Houston, TX 77058.

[1] Abbreviations: ATPase, adenosinetriphosphatase; NTP, unspecified nucleoside 5′-triphosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; NAD^+, nicotinamide adenine dinucleotide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylenimine); DCCD, dicyclohexylcarbodiimide; pCMB, *p*-(chloromercuri)benzoate.

0006-2960/82/0421-1878$01.25/0

effect of the rat liver inhibitor on the kinetics of ATP hydrolysis conducted in the presence of bicarbonate. Third, in view of conflicting reports (Pullman & Monroy, 1963; Asami et al., 1970; Lang & Racker, 1974; Chan & Barbour, 1976; Galante et al., 1981) regarding the capacity of ATPase peptide inhibitors to inhibit activities catalyzed or supported by H^+-ATPases, like the ATP-P_i exchange reaction (Pullman & Monroy, 1963; Chan & Barbour, 1976; Galante et al., 1981) and the ATP-dependent transhydrogenation of $NADP^+$ by NADH (Pullman & Monroy, 1963; Asami et al., 1970; Lang & Racker, 1974; Galante et al., 1981), it seemed important to focus on this problem as well. As indicated below, these three objectives have been met and therefore provide additional insight into the mode of action of ATPase peptide inhibitors.

Experimental Procedures

Materials

Adult, male CD^R albino rats were obtained from the Charles River Breeding Laboratories, Wilmington, MA, and were fed ad libitum a RMH 1000 rat diet manufactured by Agway, Inc., Syracuse, NY. Sucrose, D(+)-mannitol, and sodium succinate were from Baker Chemical Co. Nucleoside triphosphates, ADP, AMP, NAD^+, NADH, and $NADP^+$ were purchased from P-L Biochemicals. Glucose-6-phosphate dehydrogenase was obtained from Boehringer-Mannheim. The following enzymes were purchased from Sigma Chemical Co.: lactic dehydrogenase, yeast hexokinase, and pyruvate kinase. Defatted bovine serum albumin and pCMB were obtained also from Sigma. The following chemicals were obtained from the indicated sources: DCCD from Schwarz/Mann; Lubrol WX from ICI Organics, Inc., Providence, RI; Hepes and dithiothreitol from Calbiochem; analytical-grade sodium dihydrogen orthophosphate from Gallard Schlesinger Chemical Manufacturing Corp.; Sephadex G-75 from Pharmacia; washed and ignited sea sand from VWR Scientific, Inc. $H_3{}^{32}PO_4$ was obtained from New England Nuclear. The $^{32}P_i$ was heated in a boiling water bath for 3 h in 1.0 N HCl prior to use. Thin-layer sheets of poly(ethylenimine)-impregnated cellulose (Polygram Cel PE 1) were purchased from Brinkmann, Inc. All other reagents were of reagent-grade purity.

Methods

Isolation of Mitochondria. Rat liver mitochondria were isolated by the high-yield procedure of Bustamante et al. (1977).

Inner Membrane Vesicles. "Lubrol" inner membrane vesicles free of outer membrane, matrix, and intermembrane space activities were prepared by sequential fractionation of rat liver mitochondria with digitonin and Lubrol WX as described by Chan et al. (1970), with one minor modification. After the Lubrol treatment, and prior to harvesting the inner membrane at high speed, an additional centrifugation step at low speed (10000*g*; 10 min) was performed to remove intact mitoplasts. This yielded an inner membrane preparation with a higher ATPase specific activity than previously obtained (2.9–4.5 vs. 2.0–2.5 μmol of ATP hydrolyzed min^{-1} mg^{-1}). The inner membrane vesicles were stored in liquid nitrogen at a protein concentration of 10–20 mg/mL.

"Sonic" inner membrane vesicles also free of outer membrane, matrix, and intracristal space activities were prepared by sonication in water of water-washed mitoplast as described by Hackenbrock & Hammon (1975). The vesicles were stored in liquid nitrogen at a protein concentration of 10–20 mg/mL.

ATPase peptide inhibitor was prepared from rat liver mitochondria exactly as described previously by Cintrón & Pedersen (1979). For the final purification step to work as described, it is essential that the Sephadex G-75 be packed on top of a 1–10-cm layer of sea sand. The purified inhibitor protein has a specific activity near 5000 units/mg, where 1 unit of ATPase inhibitor activity is defined as that amount of inhibitor (milligrams) required to produce 50% inhibition of 0.2 unit of particulate ATPase activity under the specified assay conditions (Cintrón & Pedersen, 1979).

ATPase Peptide Inhibitor and ATPase Activity Assays. The assay for ATPase inhibitor activity was similar to that described by Horstman & Racker (1970). Rat liver Lubrol inner membrane vesicles (50 μg of protein; specific ATPase activity 3.5–4.6 μmol of ADP formed min^{-1} mg^{-1}) were incubated prior to the ATPase assay for 10 min at room temperature in the presence of 4 mM Tris–Hepes (pH 6.7) and 0.5 mM ATP–0.5 mM $MgCl_2$ (pH 7.5). The final volume was adjusted to 0.25 mL with 0.25 M sucrose. The pH of the prior incubation unless otherwise indicated was 6.7 ± 0.1. Mg^{2+}–ATP was always the last component added. A control without inhibitor protein was always present.

At the end of the prior incubation period, a 10-μL aliquot was transferred to an ATP-regenerating ATPase assay mixture similar to that described by Pullman et al. (1960). ATPase activity was measured spectrophotometrically at 30 °C by coupling the hydrolysis of ATP to the decrease in absorbance of NADH at 340 nm. A final volume of 1 mL contained, at pH 7.5, 58 mM Tris-HCl, 4.7 mM KCN, 4.0 mM $MgCl_2$, 4.0 mM ATP, 0.5 mM phosphoenolpyruvate, 0.3 mM NADH, 1 unit of lactate dehydrogenase, and 8 units of pyruvate kinase.

ATPase activity in the prior incubation was monitored by following the release of inorganic phosphate. Phosphate was determined by the colorimetric method of Gomori (1942).

Assay of Energy-Driven Reactions. The ATP-dependent and respiratory chain-driven nicotinamide-nucleotide transhydrogenase and the ATP-dependent succinate-linked NAD^+ reduction were all assayed by procedures similar to those described by Danielson & Ernster (1963) with some modifications. To obtain a maximal inhibitory effect and to directly compare inhibitor sensitivity of the reactions relative to the membrane-ATPase activity, we incubated varying amounts of inhibitor fraction prior to assay, under the standard conditions described above, with the only difference being that 100 μg of sonic inner membrane vesicles was employed instead of the Lubrol preparation. The prior incubations were all carried out in 1-mL cuvettes at room temperature, and all assays were performed at 30 °C. Controls without inhibitor protein were always present. The various activities were then measured as detailed below.

Assay of ATP-Dependent Transhydrogenase Activity. ATP-dependent transhydrogenase activity was assayed in the presence of an NADH-regenerating system. At the end of the prior incubation, 0.89 mL of reaction mixture (minus ATP) was added to the cuvette. After 1 min, the reaction was started by the addition of ATP. A final volume of 1.15 mL contained, at pH 7.5, 32.5 mM Tris-HCl, 3.9 mM $MgCl_2$, 162 mM sucrose, 43 mM ethanol, 0.9 mM potassium cyanide, 0.034 mM NAD^+, 0.2 mM $NADP^+$, 0.1 mg of alcohol dehydrogenase, and 1.7 mM ATP. All assay components, except ATP, were added together in a separate test tube prior to the start of the prior incubation, allowing sufficient time for the conversion of NAD^+ to NADH by alcohol dehydrogenase. The increase in absorbance at 340 nm associated with the formation of NADPH was monitored with a Gilford spectrophotometer.

The determination of the rate of the ATP-dependent reaction required a small correction for the non-energy-linked reaction, measured by omitting ATP in the assay. The inhibitor peptide had no effect on this non-energy-linked component.

Assay of Respiratory Chain Linked Transhydrogenase Activity. The assay was identical with that described above for the ATP-dependent reaction, with the following modifications: potassium cyanide was omitted from the reaction mixture, and 1 μg of oligomycin, 1.7 μM rotenone, and 2.5 mM succinate were included. The reaction was started with succinate instead of ATP.

Assay of ATP-Dependent Succinate-Linked NAD^+ Reduction Activity. After 10 min, the prior incubation was ended by sequential addition of 0.75 mL of buffer mixture, potassium cyanide, NAD^+, and succinate. After 1 min, the assay was started by the addition of ATP. In a final volume of 1.08 mL, at pH 7.5, the concentrations of the various components were as follows: 34.7 mM Tris-HCl, 4.2 mM $MgCl_2$, 174 mM sucrose, 0.9 mM potassium cyanide, 0.2 mM NAD^+, 2.8 mM succinate, and 1.9 mM ATP. The reduction of NAD^+ was followed spectrometrically at 340 nm with a Gilford spectrophotometer.

Assay of ATP–P_i Exchange Activity. Sonic inner membrane vesicles (100 μg) were first subjected to prior incubation in a series of tubes with varying amounts of inhibitor fraction. At the end of 10 min, 0.275 mL of assay mixture was added to the tubes and placed in a 30 °C water bath. After 3 min the reaction was started by the addition of ATP. A final volume of 0.6 mL contained 67 mM Tris-HCl, 4 mM sodium phosphate, 0.8 μCi of ^{32}P-labeled orthophosphate, 6.7 mM $MgCl_2$, 4 mM ADP, and 5 mM ATP. The final pH was 7.5. After incubation for 5 min, the reaction was stopped with 0.10 mL of ice-cold 2.5 N $HClO_4$, and the tubes were placed on ice. After 15 min the mixtures were neutralized with KOH and subjected to brief centrifugation to remove the precipitate. Analysis of samples and determination of exchange rates were performed as described by Pedersen (1976). Aliquots (5 μL) of each reaction mixture were spotted on PEI–cellulose thin-layer sheets, and ATP was separated from ADP, AMP, and P_i. ATP spots were cut out, and radioactivity was determined. The inhibitor produces a significant inhibition of the ATPase during the exchange assay, and as a result, the substrate concentrations become variable from sample to sample. To correct this variability, we measured the amounts of remaining phosphate and ATP for each reaction sample and incorporated them into the equation for the calculation of exchange rates (Boyer, 1959).

Assay of ATP Synthesis. Oxidative phosphorylation was assayed spectrophotometrically in the presence of an ADP-regenerating system similar to the procedure described by Pullman & Racker (1956). An initial standard prior incubation of sonic particles (100 μg) with the inhibitor fraction was performed as described above in a 1-mL cuvette. The prior incubation was ended by the addition of 0.75 mL of reaction mixture. After 3 min ADP was added to start the reaction. In a final volume of 1.0 mL, at pH 7.5, the concentrations of the assay components were 37.5 mM Tris–acetate, 0.75 mM glucose, 7.4 mM succinate, 0.75 mM $NADP^+$, 7.5 mM potassium phosphate, 1.5 mM $MgCl_2$, 30 μg of hexokinase, 15 μg of glucose-6-phosphate dehydrogenase, 2 mM AMP, and 1 mM ADP. The reaction was carried out at 30 °C. All the reaction components (except ADP) were mixed together prior to the start of the assay. AMP was included in the assay to minimize the rate of contaminating adenylate kinase, reducing it to less than 2% of the rate of oxidative phosphorylation. The small amount of ATP remaining from the prior incubation step is converted to ADP by the system during the 3 min prior to the addition of exogenous ADP. The increase of absorbance at 340 nm associated with the formation of NADPH was followed with a Gilford spectrophotometer. The reaction was linear for more than 10 min.

Determination of Protein. Membrane protein was estimated by the biuret reaction in the presence of 0.33% sodium cholate (Jacobs et al., 1956). Bovine serum albumin was routinely used as the standard in all cases.

Table I: Nucleotide Requirement in the Prior Incubation Mixture Effective in Supporting Peptide Inhibitor-H^+-ATPase Interaction[a]

prior incubn component	ATPase act. (μmol of ATP hydrolyzed min^{-1} mg^{-1}) without inhibitor	ATPase act. (μmol of ATP hydrolyzed min^{-1} mg^{-1}) with inhibitor	inhibition (%)
ATP, $MgCl_2$	3.66	0.61	83
ITP, $MgCl_2$	3.54	0.86	76
GTP, $MgCl_2$	3.90	1.50	62
ADP, $MgCl_2$	3.22	2.98	8
P_i, $MgCl_2$	3.30	3.30	0
ADP, P_i, $MgCl_2$	3.30	3.16	4
ATP, EDTA	3.38	2.95	13
ITP, EDTA	2.90	2.95	0
GTP, EDTA	3.06	3.14	0
ADP, EDTA	3.14	3.22	0
P_i, EDTA	3.66	3.38	7

[a] Lubrol inner membrane vesicles (50 μg) were incubated with purified ATPase inhibitor peptide (0.44 μg) for 10 min at 25 °C with the indicated compounds. Prior incubation conditions were exactly as described under Methods. The final prior incubation concentrations of NTP, ADP, $MgCl_2$, and EDTA when present were 0.5 mM. Results represent averages of triplicate determinations.

Results

Requirement for Prior Incubation To Support H^+–ATPase–Peptide Inhibitor Interactions. Studies by Pullman & Monroy (1963) and Horstman & Racker (1970) provided detailed information about the prior incubation conditions required to promote optimal interactions between the ATPase inhibitor peptide of bovine heart mitochondria and the H^+–ATPase associated with submitochondrial particles from the same source. These workers emphasized the need for ATP and Mg^{2+} in the prior incubation and showed that the optimal pH for interaction was slightly below 7. As indicated in our previous communication on the ATPase peptide inhibitor of rat liver mitochondria (Cintrón & Pedersen, 1979), similar prior incubation conditions support optimal interactions between the rat liver inhibitor and the H^+–ATPase associated with purified mitochondrial inner membrane vesicles from the same source. In this study we have examined the prior incubation conditions in greater detail with the purpose of establishing *why* these conditions are essential to support optimal H^+–ATPase–peptide interactions.

Results presented in Table I show that in addition to ATP and Mg^{2+}, ITP and GTP also support H^+–ATPase–inhibitor interactions provided Mg^{2+} is present. ITP and GTP are both substrates for the H^+–ATPase of rat liver and are hydrolyzed almost as readily as ATP, provided Mg^{2+} is present (Pedersen, 1976). Removal of Mg^{2+} from either ATP, ITP, or GTP by complexation with EDTA renders these triphosphates ineffective in supporting optimal H^+–ATPase–inhibitor interac-

Table II: Dependence of H^+-ATPase-Peptide Inhibitor Interactions on ATP Hydrolysis [a]

Part A	
components added to prior incubn mixture	ATP hydrolyzed in prior incubn [b]
inner membrane vesicles	118
+oligomycin	26
+DCCD	38
+EDTA	26
+EDTA + $MgCl_2$	118
Part B	
components added to prior incubn mixture	ATPase act. following prior incubn [c]
inner membrane vesicles	3200
+oligomycin	39
+DCCD	49
+inhibitor peptide	55
+EDTA + inhibitor peptide	3050
+EDTA + inhibitor peptide + $MgCl_2$	55

[a] Lubrol inner membrane vesicles (0.25 mg) were incubated 5 min prior to assay in a total volume of 0.25 mL containing 0.5 mM ATP, 0.5 mM $MgCl_2$, and 4 mM Tris-Hepes, pH 6.7, and where indicated 0.3 μg of oligomycin, 20 μM DCCD, 2 μg of ATPase inhibitor peptide, 5.0 mM EDTA, or 5.0 mM additional $MgCl_2$. In part A, the reaction was terminated with 0.025 mL of 2.5 M PCA and the entire reaction mixture subjected to P_i analysis according to the procedure of Gomori (1942). The values presented were corrected for small amounts of P_i contaminating the inner membrane vesicles and ATP. In part B, 2 μL of the prior incubation mixture was withdrawn after 5 min and assayed for ATP hydrolytic activity by the spectrophotometric procedure described under Methods. When additional $MgCl_2$ was added to the prior incubation mixture, incubation was carried out for an additional 5 min. Results represent averages of triplicate determinations. [b] In units of nanomoles of P_i released in 5 min. [c] In units of ATP hydrolyzed per minute per milligram.

tions. ADP and P_i either separately or together (or in the presence of Mg^{2+}) also fail to support H^+-ATPase–inhibitor interactions.

Dependence of H^+-ATPase–Peptide Inhibitor Interactions on ATP Hydrolysis. Results presented in Table IIA show that under the prior incubation conditions used in these studies a significant amount of the ATP present is hydrolyzed. This ATP hydrolytic activity can be markedly inhibited by both oligomycin and DCCD, confirming the involvement of the H^+-ATPase. Addition of 5.0 mM EDTA to complex the Mg^{2+} is equally as effective as oligomycin or DCCD in suppressing the ATP hydrolytic activity. Table IIA shows also that the inhibitory effect of EDTA can be readily reversed by addition of excess $MgCl_2$.

Results presented in Table IIB show specific activities of the H^+-ATPase after undergoing prior incubation with and without the peptide inhibitor in the presence and absence of EDTA. When EDTA is absent, the peptide inhibitor is almost as effective as oligomycin and DCCD in suppressing the ATP hydrolytic activity of the H^+-ATPase. If EDTA is present in the prior incubation mixture to complex Mg^{2+}, the inhibitor peptide has little effect on the capacity of the H^+-ATPase to catalyze ATP hydrolysis. Addition of sufficient Mg^{2+} to reverse the EDTA effect in the prior incubation (and therefore to replenish Mg^{2+}-ATP and reinitiate ATP hydrolytic activity) restores the capacity of the peptide inhibitor to inhibit the H^+-ATPase. Therefore, results presented in Table II indicate

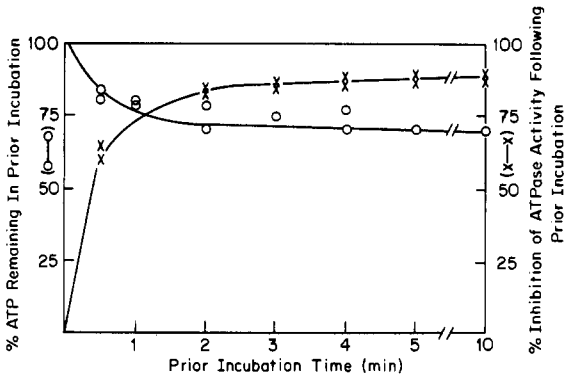

FIGURE 1: ATPase activity in the prior incubation and inhibition of H^+-ATPase activity: relative time courses. Prior incubation conditions were exactly as described in Table II with 2 μg of ATPase peptide inhibitor present. At the indicated time points, the reaction mixture was assayed both for P_i (Gomori, 1942) to monitor ATP disappearance and for ATPase activity to monitor the effect of the inhibitor peptide. The latter assay was carried out with the spectrophotometric procedure described under Methods. The initial amount of ATP in the prior incubation corresponding to 100% was 125 nmol. The specific ATPase activity in the incubation corresponding to 100% was 3.5 μmol of ATP hydrolyzed min^{-1} mg^{-1}.

that the formation of an inactive H^+-ATPase–peptide inhibitor complex is dependent on ATP hydrolysis.

ATPase Activity in the Prior Incubation and Inhibition of H^+-ATPase Activity: Relative Time Courses. Figure 1 shows that the disappearance of ATP in the preliminary incubation correlates well with the onset of inhibition of H^+-ATPase activity. In about 3 min near maximal inhibition of the ATPase activity is reached, a time at which ATP levels in the preliminary incubation mixture become constant. In the experiment shown, 125 nmol of ATP was present initially in the preliminary incubation. Hydrolysis of about 30% of the initial ATP or 38 nmol is required to effect maximal inhibition of the H^+-ATPase (by its peptide inhibitor) in 50 μg of inner membrane vesicles.

Effect of the Inhibitor Peptide on the Kinetics of ATP Hydrolysis Catalyzed by the Rat Liver H^+-ATPase. It has been established in previous studies in Lardy's laboratory (Ebel & Lardy, 1975a,b; Lardy et al., 1975) and in this laboratory (Pedersen, 1976) that the ATP hydrolytic activity of the liver H^+-ATPase is characterized by typical Michaelis–Menten kinetics when assayed in Tris–HCO_3 buffer and biphasic kinetic patterns when assayed in Tris-HCl buffer. HCO_3^- is an activator of ATP hydrolytic activity relative to Cl^-. Unlike ATP hydrolysis, the hydrolysis of ITP and GTP are characterized by typical Michaelis–Menten kinetics in both Tris-HCl and Tris–HCO_3 buffers (Ebel & Lardy, 1975b; Pedersen, 1976). These and other results (Ebel & Lardy, 1975a,b; Lardy et al., 1975; Pedersen, 1976) have been interpreted to indicate that the liver H^+-ATPase is characterized by at least one allosteric site each for ATP (a negative effector) and bicarbonate (a positive effector) in addition to the hydrolytic site(s) (Lardy et al., 1975; Pedersen, 1976). Results presented in Figure 2A confirm these previous findings and show that the H^+-ATPase present in purified inner membrane vesicles of rat liver mitochondria used in this study also exhibits monophasic kinetic patterns in Tris–HCO_3 buffer and biphasic patterns in Tris-HCl buffer. It is clear in Figure 2A that HCO_3^- is an activator relative to Cl^-. The biphasic character of the kinetic patterns observed in Tris-HCl buffer is seen more clearly in the Eadie–Hofstee plots presented in Figure 2B.

The effect of the ATPase peptide inhibitor on the kinetics of ATP hydrolysis catalyzed by the liver H^+-ATPase in both Tris-HCl and Tris–HCO_3 buffer is presented in Figure 3 and

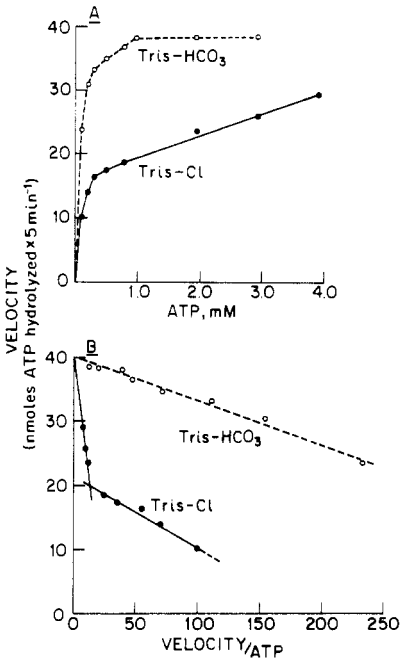

FIGURE 2: ATPase activity of inner membrane vesicles as a function of ATP concentration. Lubrol inner membrane vesicles (50 μg) were incubated prior to the ATPase activity assay by using the standard conditions described under Methods. After 10 min, a 10-μL aliquot (2 μg of membranes) was transferred to the ATPase assay system described under Methods containing as buffer either 65 mM Tris-HCl, pH 7.5, or 65 mM Tris–HCO_3, pH 7.5. ATPase activity was determined at concentrations of Mg^{2+}–ATP between 0.1 and 4.0 mM. Kinetic data are presented as (A) velocity vs. ATP concentration plots and (B) Eadie–Hofstee plots (Eadie, 1952; Hofstee, 1952) with ATP concentrations in millimolar.

Table III: Effect of Purified ATPase Inhibitor Peptide on the Apparent Kinetic Constants of Inner Membrane H^+-ATPase [a]

	in Tris-HCl buffer		in Tris-HCO_3 buffer	
assay condition	K_m [b]	V_{max} [c]	K_m [b]	V_{max} [c]
control (without inhibitor)	0.15, 0.87	2750, 4053	0.043	4010
with inhibitor (0.22 μg)	0.13, 0.86	1000, 1670	0.057, 0.16	1950, 2313

[a] Apparent kinetic constants were calculated from the Eadie–Hofstee plots (Eadie, 1952; Hofstee, 1952) presented in Figure 3. The extrapolated values on the ordinates are V_{max}, and the extrapolated values on the abscissas are V_{max}/K_m. V_{max} values are presented as the values extrapolated from the ordinates divided by the milligrams of inner membrane protein in the ATPase assay and therefore represent the maximal ATPase specific activities under the assay conditions described. [b] In units of millimolar. [c] In units of nanomoles per minute per milligram.

in Table III. When assayed in Tris-HCl buffer, the peptide inhibitor has the characteristics of a noncompetitive inhibitor in that it lowers the V_{max} of the enzyme but has little effect on the apparent K_ms for ATP extrapolated from the two phases of the Eadie–Hofstee plots (Table III). Significantly, when the H^+–ATPase is assayed in Tris–HCO_3 buffer, the inhibitor peptide is seen to convert the typical monophasic Michaelis–Menten pattern to a biphasic pattern.

These results indicate that the activating anion HCO_3^- and the ATPase peptide inhibitor have opposing actions. The inhibitor peptide appears to unmask an ATP site (most likely an allosteric site), which remains functionally silent in the presence of HCO_3^-.

Effect of the Inhibitor Peptide on Energy-Linked Reactions Catalyzed or Supported by the Rat Liver H^+–ATPase. Because the ATPase inhibitor inhibits the ATPase activity of

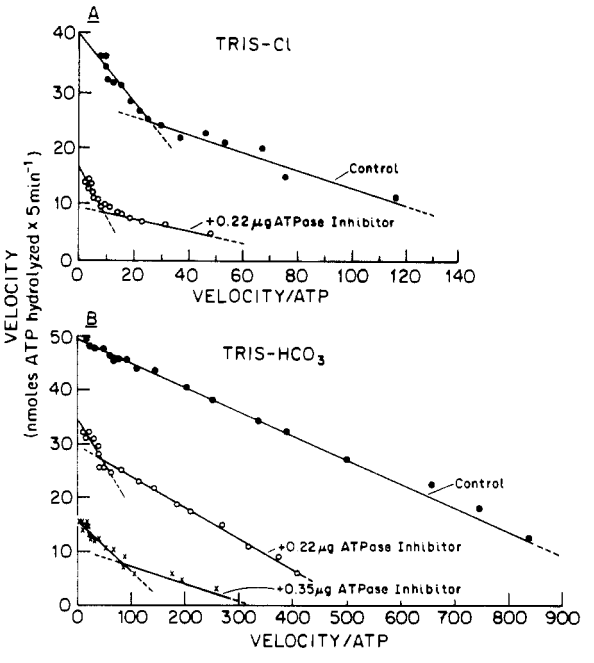

FIGURE 3: Effect of the ATPase inhibitor peptide on the kinetics of the ATPase reaction catalyzed by Lubrol inner membrane vesicles in Tris-HCl or Tris–HCO_3 buffer. (A) Assayed in Tris-HCl buffer. Lubrol inner membrane vesicles (50 μg) were incubated prior to the ATPase activity assay in the absence or presence of the purified ATPase inhibitor (0.22 μg) under the conditions described under Methods. After prior incubation, a 10-μL aliquot (2 μg of membranes) was transferred to the ATPase assay system described under Methods containing 65 mM Tris-HCl buffer at pH 7.5. ATPase activity was determined at concentrations of Mg^{2+}–ATP between 0.1 and 4.5 mM. Kinetic data are presented as Eadie–Hofstee plots (Eadie, 1952; Hofstee, 1952) with ATP concentrations in millimolar. (●) Kinetic plot obtained in the absence of ATPase inhibitor; (○) kinetic plot obtained in the presence of purified ATPase inhibitor. (B) Assayed in Tris–HCO_3 buffer. Conditions and symbols are as in (A) with the following exceptions. Lubrol inner membrane vesicles were present at 75 μg in the prior incubation assay, and 3 μg was taken for assay. The Tris-HCl buffer was replaced by Tris–HCO_3 buffer (65 mM; pH 7.5) in the assay medium.

H^+–ATPases, it seems reasonable to assume that it might also suppress the activities of all ATP-dependent activities catalyzed or supported by such enzymes. However, there is not agreement in the literature regarding this point. Some investigators working with the bovine heart enzyme have failed to find inhibition of the ATP-dependent transhydrogenase reaction (Pullman & Monroy, 1963; Lang & Racker, 1974) whereas other investigators report marked inhibitions of the same reaction (Asami et al., 1970; Ferguson et al., 1977). In the case of the rat liver inhibitor, Chan & Barbour (1976) report that an inhibitor preparation with a specific activity of 700 units (about $^1/_7$ the specific activity of the purified liver preparation used in this study) has little effect on the ATP–P_i exchange reaction catalyzed by submitochondrial particles of rat liver.

With the above background information in mind, we first examined the effect of a partially purified preparation of the rat liver inhibitor peptide, comparable in specific activity to that used by Chan & Barbour (1976), on several ATP-dependent activities and on ATP synthesis. In these studies we used as a source of H^+–ATPase sonic inner membrane vesicles because they are >90% inverted (Wehrle et al., 1978) and catalyze both ATP-dependent activities and ATP synthesis at high rates (Pedersen & Hullihen, 1978). Figure 4A shows that the partially purified rat liver inhibitor peptide almost completely inhibits, in addition to ATPase activity, the ATP-dependent transhydrogenase reaction and the ATP-dependent, succinate-linked, NAD^+ reduction reaction. When

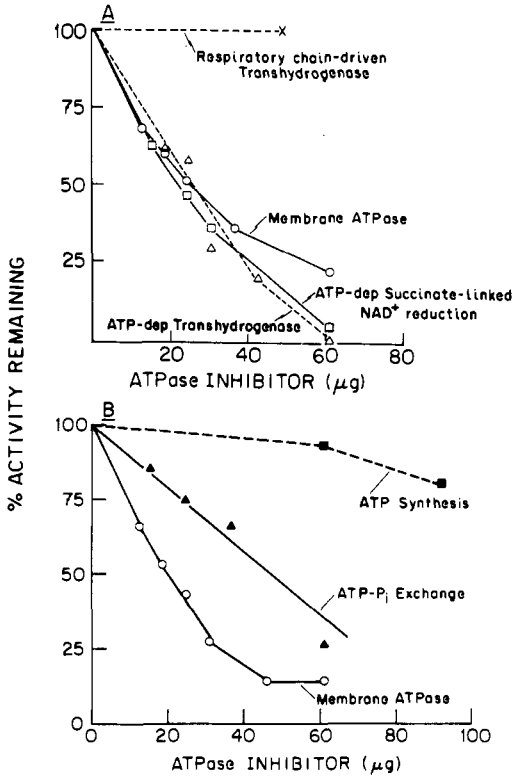

FIGURE 4: Effect of partially purified ATPase inhibitor peptide on energy-linked activities of inner membrane vesicles. Prior incubation of sonic vesicles (100 μg) with ATPase inhibitor purified through the heat step (Cintrôn & Pedersen, 1979) and assays of the various energy-linked activities were carried out exactly as described under Methods. (A) Activities in the absence of ATPase inhibitor were 3.3 μmol of ATP hydrolyzed min^{-1} mg^{-1} for ATPase (○), 101 nmol of NADH formed min^{-1} mg^{-1} for ATP-dependent succinate-linked NAD^+ reduction (□), 72 nmol of NADH formed min^{-1} mg^{-1} for ATP-dependent transhydrogenase (△), and 102 nmol of NADH formed min^{-1} mg^{-1} for respiratory chain driven transhydrogenase (×). (B) Activities in the absence of ATPase inhibitor were 63 nmol of ATP formed min^{-1} mg^{-1} for oxidative phosphorylation (□), 171 nmol of ATP exchanged min^{-1} mg^{-1} for the ATP–P_i exchange reaction (▲), and 3.3 μmol of ATP hydrolyzed min^{-1} mg^{-1} for ATPase (○).

the transhydrogenase reaction is driven by substrate oxidation rather than by ATP, it is unaffected by the inhibitor peptide. Figure 4B shows that the ATP–P_i exchange reaction is markedly inhibited also by the partially purified inhibitor preparation but requires almost twice as much inhibitor to effect 50% inhibition as do the other ATP-dependent reactions tested. The steady-state rate of ATP synthesis is unaffected by titers of the inhibitor preparation that maximally suppress ATP-dependent activities. Finally, results presented in Table IV show that homogeneous peptide inhibitor also suppresses all ATP-dependent activities tested, including the ATP–P_i exchange reaction, but is without effect on the steady-state rate of ATP synthesis.

These results show that the rat liver ATPase peptide inhibitor is a potent inhibitor of all ATP-dependent activities tested but has little effect on the steady-state rate of ATP synthesis. Whether or not a given ATP-dependent activity is inhibited is independent of the degree of purity of the preparation but very dependent on the amount of inhibitor used.

Discussion

Results summarized in this report provide the first information documented to date about the interaction of the homogeneous rat liver ATPase peptide inhibitor with the H+-ATPase associated with purified, inverted inner membrane vesicles from the same source. The experiments were designed to address three questions, the answers to which are fundamental to our eventual understanding of the potential regulatory role of the inhibitory peptide. Each of these questions has been answered either completely or in part and will be dealt with separately.

Table IV: Effect of Purified ATPase Inhibitor Peptide on Energy-Associated Reactions of Inner Membrane Vesicles[a]

act. measured	sp act. (nmol of product formed min^{-1} mg^{-1}) without inhibitor	sp act. (nmol of product formed min^{-1} mg^{-1}) with inhibitor	inhibition (%)
ATPase	4063	1063	73
ATP-dependent NADH–$NADP^+$ transhydrogenase	124	40	67
ATP-dependent succinate-linked NAD^+ reduction	129	49	62
ATP–P_i exchange	248	65	74
ATP synthesis	191	201	0

[a] Prior incubation of sonic inner membrane vesicles (50 μg) with the purified ATPase inhibitor and assays of the indicated energy-linked activities were exactly as described under Methods. Where indicated, 0.3 μg of ATPase inhibitor peptide was present except in the case of the ATP–P_i exchange reaction where 0.5 μg was used. Results represent averages of triplicate determinations.

The first question focused on elucidating the role of ATP and Mg^{2+} in the prior incubation mixture. Earlier studies on the bovine heart inhibitor by Pullman & Monroy (1963) and Horstman & Racker (1970) emphasized the necessity for prior incubation in the presence of ATP and Mg^{2+} to effect inhibition of the ATPase activity of both membrane-bound and purified F_1–ATPase. In extending these studies, Gomez-Fernandez & Harris (1978) showed that a variety of nucleoside triphosphates, all of which are hydrolytic substrates for the bovine heart H+-ATPase, would support inhibition of the enzyme when included in the prior incubation. In this report we show that ATP hydrolysis does take place in the prior incubation mixture (Table II), that the hydrolytic reaction correlates with the inhibition of the H+-ATPase by its inhibitor peptide (Figure 1), that EDTA prevents both this inhibition and ATP hydrolysis in the prior incubation (Table II), and that ATP cannot be replaced in the prior incubation by ADP and P_i (Table I). These results strongly suggest that ATP hydrolysis may be necessary to effect formation of an inactive H+-ATPase–inhibitor complex.

The finding that ADP and P_i cannot replace ATP in the prior incubation mixture could be taken to suggest that ATP hydrolysis is essential to effect energization of the H+-ATPase and in some way facilitate its interaction with the peptide inhibitor. We believe energization by a single turnover of ATP to be unlikely because many more molecules of ATP are hydrolyzed than H+-ATPase molecules inhibited. As indicated in the text, about 38 nmol of ATP is hydrolyzed in the time required to effect maximal inhibition of the H+-ATPase associated with 50 μg of inner membrane vesicles. The minimal amount of F_1–ATPase associated with inner membrane vesicles has been estimated to be 10% of the total mass (Pedersen et al., 1978), and the molecular weight of the rat liver enzyme is 384 000 (Catterall & Pedersen, 1971). Therefore, roughly 2800 molecules of ATP are required to effect maximal inhibition of 1 molecule of F_1–ATPase. A more rational explanation of the data is that the inhibitor peptide (in its functional mode) interacts with a form of the enzyme that has bound not only the substrate Mg^{2+}–ATP but one or both of the products ADP and P_i, as indicated in Figure 5A. Along

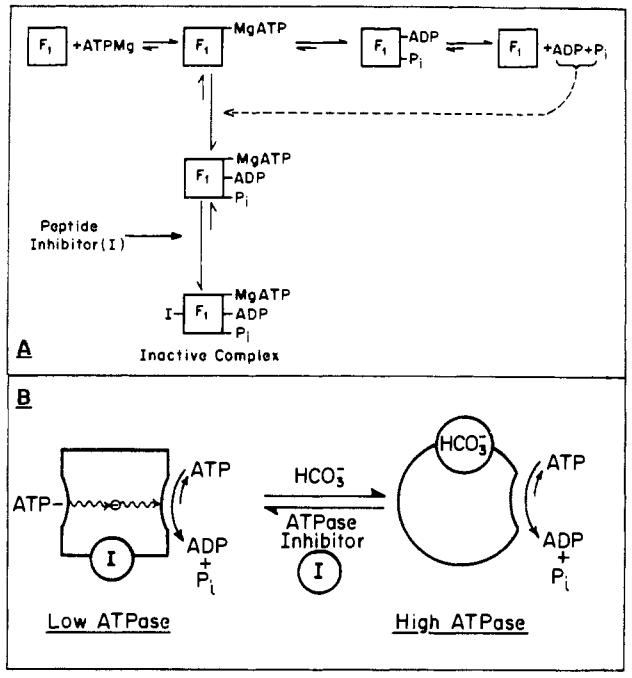

FIGURE 5: Schemes summarizing H^+–ATPase (F_0F_1) and ATPase peptide inhibitor interactions. The scheme summarized in (A) indicates that the ATPase peptide inhibitor of rat liver may interact with a transient form of the H^+–ATPase of rat liver (see Discussion for justification). The scheme summarized in (B) accounts for kinetic observations made when H^+–ATPase is assayed in the presence and absence of bicarbonate buffer with and without peptide inhibitor. Bicarbonate is depicted as favoring the formation of a high ATPase activity form of the H^+–ATPase free from allosteric inhibition by ATP. The peptide inhibitor, on the other hand, is depicted as favoring a form of the H^+–ATPase in which ATP operating through an allosteric site negatively affects the hydrolytic site.

these lines it is interesting to note that Gomez-Fernandez & Harris (1978) suggested that the bovine heart ATPase peptide inhibitor may interact with a transient form of the bovine heart H^+–ATPase. It is also relevant to this discussion that Galante et al. (1981) have suggested that inhibitor peptide may already be bound to the bovine heart H^+–ATPase in the membrane prior to ATP hydrolysis, with the hydrolytic reaction converting the complex to an enzymatically inhibited state.

The second question addressed in this study dealt with establishing the effect of the rat liver ATPase inhibitor peptide on the kinetics of ATP hydrolysis catalyzed by the H^+–ATPase from the same source. The central focus of this aspect of the study was to ascertain whether the inhibitor peptide is of the competitive or noncompetitive type and whether the activating anion bicarbonate opposed the action of the inhibitor. These studies revealed that the inhibitor peptide is noncompetitive with respect to the substrate ATP (Figure 3, Table III) and that in the presence of bicarbonate it appears to induce the operation of at least one ATP site, which normally is functionally silent in the presence of this activating anion (Figure 3). These data, taken together with other kinetic data presented here, are consistent with the view that two states of the H^+–ATPase can exist in solution (Figure 5B). One state is viewed as being stabilized by bicarbonate such that at least one ATP site is functionally silent. The other state is viewed as being stabilized by the inhibitor peptide with at least one ATP site expressed as a negative effector of the ATP hydrolytic site. The model depicts the inhibitor binding site as being distinct from both the ATP allosteric and substrate binding sites because the inhibitor was found to have little effect on the apparent K_ms (ATP) extrapolated from the two phases of the kinetic plots shown in Figure 3 (see also Table III).

The third and final question addressed in this study focused on ascertaining the capacity of the rat liver ATPase peptide inhibitor to inhibit ATP-dependent activities catalyzed by the H^+–ATPase associated with purified, inverted inner membrane vesicles from the same source. In contrast to some earlier reports with the purified bovine heart peptide inhibitor (Pullman & Monroy, 1963; Lang & Racker, 1974) and a partially purified preparation of the liver peptide inhibitor (Chan & Barbour, 1976), it was found that either a partially purified (Figure 4) or a homogeneous preparation of the rat liver peptide inhibitor (Table IV) markedly suppresses all ATP-dependent activities examined. The ATP–P_i exchange reaction was found to require almost 2-fold higher amounts of inhibitor peptide to effect 50% inhibition than the other ATP-dependent activities tested. Consistent with this result, Galante et al. (1981) report that the ATP–P_i exchange activity of complex V from bovine heart can be inhibited as much as 50% by the bovine heart inhibitor peptide. This finding may account for the failure of Chan & Barbour (1976) to find an effect of the rat liver inhibitor peptide on this activity. Since all ATP-dependent activities catalyzed by the H^+–ATPase are usually thought to reflect, at least in part, the ATPase activity catalyzed by the enzyme, it seems mechanistically consistent that all such activities are inhibited also by the peptide inhibitor.

The finding that the ATPase peptide inhibitor of rat liver mitochondria has little or no effect on the steady-state rate of ATP synthesis (Figure 4; Table IV) has been observed previously with the inhibitor peptide of bovine heart mitochondria (Pullman & Monroy, 1963; Asami et al., 1970). In terms of function, it would appear therefore that the ATPase peptide inhibitor operates predominantly as a unidirectional inhibitor. From a physiological point of view, this seems reasonable since newly synthesized ATP would be protected from hydrolysis when the energy source derived from respiration either slows down or terminates altogether. From a mechanistic point of view, however, there appears to be a paradox because an inhibitor of the reverse direction of an enzymatic reaction (in this case ATP hydrolysis) would be predicted to inhibit also the forward direction of the reaction (in this case ATP synthesis). To resolve this apparent paradox, we have carried out a separate study, the details of which are summarized in another report (Schwerzmann & Pedersen, 1981).

Acknowledgments

We are grateful to Drs. Janna Wehrle, Mario Amzel, Ernesto Bustamante, and Naomi Geller-Lipsky for helpful suggestions while this work was in progress.

References

Asami, K., Juntti, K., & Ernster, L. (1970) *Biochim. Biophys. Acta 205*, 307–311.

Boyer, P. D. (1959) *Arch. Biochem. Biophys. 82*, 387–410.

Brooks, J. C., & Senior, A. E. (1971) *Arch. Biochem. Biophys. 147*, 467–470.

Burtnick, L. D., McCubbin, W. D., & Kay, C. M. (1975) *Can. J. Biochem. 52*, 1207–1213.

Bustamante, E., Soper, J. W., & Pedersen, P. L. (1977) *Anal. Biochem. 80*, 401–408.

Catterall, W. A., & Pedersen, P. L. (1971) *J. Biol. Chem. 246*, 4987–4994.

Chan, S. H. P., & Barbour, R. L. (1976) *Biochim. Biophys. Acta 430*, 426–433.

Chan, T. L., Greenawalt, J. W., & Pedersen, P. L. (1970) *J. Cell Biol. 45*, 291–305.

Cintron, N. M., & Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 3439–3443.
Danielson, L., & Ernster, L. (1963) *Biochem. Biophys. Res. Commun. 10*, 91–96.
Eadie, G. S. (1952) *Science (Washington, D.C.) 116*, 688.
Ebel, R. E., & Lardy, H. A. (1975a) *J. Biol. Chem. 250*, 191–196.
Ebel, R. E., & Lardy, H. A. (1975b) *J. Biol. Chem. 250*, 4992–4995.
Ebner, E., & Maier, K. L. (1977) *J. Biol. Chem. 252*, 671–676.
Ferguson, S. T., Harris, D. A., & Radda, G. (1977) *Biochem. J. 162*, 351–357.
Galante, Y., Wong, S.-Y., & Hatefi, Y. (1981) *Biochemistry 20*, 2671–2678.
Gomez-Fernandez, J. C., & Harris, D. A. (1978) *Biochem. J. 176*, 967–975.
Gomori, G. (1942) *J. Lab. Clin. Med. 27*, 955–960.
Greaser, M. L., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226–4233.
Hackenbrock, C. R., & Hammon, K. M. (1975) *J. Biol. Chem. 250*, 9185–9197.
Hofstee, B. H. J. (1952) *Science (Washington, D.C.) 116*, 329–331.
Horstman, L. L., & Racker, E. (1970) *J. Biol. Chem. 245*, 1336–1344.
Jacobs, E. E., Jacob, M., Sanadi, D. R., & Bradley, L. B. (1956) *J. Biol. Chem. 223*, 147–156.
Lambeth, D. O., & Lardy, H. A. (1971) *Eur. J. Biochem. 22*, 355–363.
Lang, D. R., & Racker, E. (1974) *Biochim. Biophys. Acta 333*, 180–186.
Lardy, H. A., Schuster, S. M., & Ebel, R. E. (1975) *J. Supramol. Struct. 3*, 214–221.
Mitchell, P., & Moyle, J. (1971) *Bioenergetics 2*, 1–11.
Nelson, N., Nelson, H., & Racker, E. (1972) *J. Biol. Chem. 247*, 7657–7662.
Pedersen, P. L. (1976) *J. Biol. Chem. 251*, 934–940.
Pedersen, P. L., & Hullihen, J. (1978) *J. Biol. Chem. 253*, 2176–2183.
Pedersen, P. L., Amzel, L. M., Soper, J. W., Cintrôn, N., & Hullihen, J. (1978) in *Energy Conservation in Biological Membranes* (Schäfer, G., & Klingenberg, M., Eds.) pp 158–194, Springer-Verlag, New York.
Pullman, M. E., & Racker, E. (1956) *Science (Washington, D.C.) 123*, 1105–1107.
Pullman, M. E., & Monroy, G. C. (1963) *J. Biol. Chem. 238*, 3762–3769.
Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322–3329.
Satre, M., de Jerphanion, M. B., Huet, J., & Vignais, P. V. (1975) *Biochim. Biophys. Acta 387*, 241–255.
Satre, M., Klein, G., & Vignais, P. V. (1979) *Methods Enzymol. 55*, 421–426.
Schwerzmann, K., & Pedersen, P. L. (1981) *Biochemistry 20*, 6305–6311.
Smith, J. B., & Sternweis, P. C. (1977) *Biochemistry 16*, 306–311.
Smith, J. B., Sternweis, P. C., & Heppel, L. A. (1975) *J. Supramol. Struct. 3*, 248–255.
Wehrle, J. P., Cintrôn, N. M., & Pedersen, P. L. (1978) *J. Biol. Chem. 253*, 8598–8603.
Wilkinson, J. M., Perry, S. V., Cole, H. A., & Trayer, I. P. (172) *Biochem. J. 127*, 215–228.

Electron Uptake and Delivery Sites on Plastocyanin in Its Reactions with the Photosynthetic Electron Transport System†

Ole Farver, Yosepha Shahak, and Israel Pecht*

ABSTRACT: French bean plastocyanin is stoichiometrically and specifically labeled upon reduction by $Cr(II)_{aq}$ ions, yielding a substitution-inert Cr(III) adduct at the protein surface. The effect of the modification on the activity of plastocyanin in electron transfer between photosystems II and I has been investigated. The photoreduction and photooxidation by chloroplasts or by photosystem I reaction centers, respectively, of native and Cr(III)-labeled plastocyanin have been compared. It was found that whereas the photoreduction rates of native and Cr-labeled plastocyanin were indistinguishable, the rates of photooxidation of the modified protein were markedly attenuated relative to those of the native one. This difference in reactivity clearly reflects the perturbation of the electron transfer pathway to P_{700}. These findings, in conjunction with the structure of plastocyanin and the locus of Cr(III) binding on its surface, lead to the following interpretation: (a) There are most probably two physiologically significant, electron transfer sites on plastocyanin. (b) The site involved in the electron transfer to P_{700} is most likely in the region of tyrosine-83 and the negatively charged patch proximal to it. By elimination we assume that the second site is centered at the hydrophobic region of histidine-87.

Plastocyanin (Pc)[1] is a "blue" single copper protein functioning as an electron carrier in algae and higher plants. Its site of action in the photosynthetic electron transport chain is commonly believed to be located between cytochrome *f* and P_{700} (Wessels, 1966; Gorman & Levine, 1966; Avron & Shneyour, 1971; Wood & Bendall, 1975; Haehnel et al., 1980;

† From the Departments of Chemical Immunology and Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel. *Received June 25, 1981.* O.F. received a grant from the Danish Natural Science Research Council.

[1] Abbreviations: Pc, plastocyanin; RC, photosystem I reaction centers; PS I or II, photosystem I or II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4-dinitrophenyl ether of 2-iodo-4-nitrothymol; Pc(I) or Pc(II), reduced or oxidized plastocyanin; Cr-Pc, chromium-labeled plastocyanin; cyt *f*, cytochrome *f*; NMR, nuclear magnetic resonance; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

0006-2960/82/0421-1885$01.25/0