Condon, M. D., Petrillo, E. W., Jr., Ryono, D. E., Reid, J. A., Neubeck, R., Puar, M., Heikes, J. E., Sabo, E. F., Losee, K. A., Cushman, D. W., & Ondetti, M. A. (1982) *J. Med. Chem. 25*, 250–258.

Cushman, D. W., & Ondetti, M. A. (1980) *Prog. Med. Chem. 17*, 41–104.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.

Das, M., & Soffer, R. L. (1975) *J. Biol. Chem. 250*, 6762–6768.

Fasman, G. D. (1976) *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data*, Vol. 1, pp 318, 321, CRC Press, Cleveland, OH.

Galardy, R. E. (1980) *Biochem. Biophys. Res. Commun. 97*, 94–99.

Greenstein, J. P., & Winitz, M. (1961a) *Chemistry of the Amino Acids*, Vol. 2, p 942, Wiley, New York.

Greenstein, J. P., & Winitz, M. (1961b) *Chemistry of the Amino Acids*, Vol. 3, p 2760, Wiley, New York.

Hamer, N. K., & Tack, R. D. (1974) *J. Chem. Soc., Perkin Trans. 2*, 1184–1187.

Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl Acad. Sci. U.S.A. 76*, 6216–6220.

Itoh, M., Hagiwara, D., & Kamiya, T. (1975) *Tetrahedron Lett. 49*, 4393–4394.

Kam, C. M., Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 3032–3038.

Oshima, G., & Nagasawa, K. (1976) *J. Biochem. (Tokyo) 31*, 57–63.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280–283.

Quiocho, R. A., & Lipscomb, W. N. (1971) *Adv. Protein Chem. 25*, 1–78.

Sweet, C. S., Gross, D. M., Arbegast, P. T., Gaul, S. L., Britt, P. M., Ludden, C. T., Weitz, D., & Stone, C. A. (1981) *J. Pharmacol. Exp. Ther. 216*, 558–566.

Thorsett, E. D., Harris, E. E., Peterson, E. R., Greenlee, W. J., Patchett, A. A., Ulm, E. H., & Vassil, T. C. (1982) *Proc. Natl Acad. Sci. U.S.A. 79*, 2176–2180.

Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.

Webb, J. L. (1961) *Enzyme and Metabolic Inhibitors*, Vol. I, pp 66–79, Academic Press, New York.

Equilibrium Binding of ^{125}I-Labeled Adenosinetriphosphatase Inhibitor Protein to Complex V of the Mitochondrial Oxidative Phosphorylation System†

Siu-Yin Wong, Yves M. Galante, and Youssef Hatefi*

ABSTRACT: The ATPase inhibitor protein (IF_1) purified from bovine heart mitochondria was labeled with ^{125}I at its single tyrosyl residue under conditions that inhibitor potency was fully preserved. Equilibrium binding studies of [^{125}I]IF_1 to complex V (purified ATP synthetase complex) under conditions favoring inhibition revealed the presence in complex V of saturable high-affinity as well as low-affinity binding sites for [^{125}I]IF_1. The double-reciprocal plot of data concerned with [^{125}I]IF_1 binding to the complex V high-affinity site as a function of added [^{125}I]IF_1 indicated a saturation point of 0.94 mol of [^{125}I]IF_1 bound per mol of complex V and a dissociation constant of 0.75 μM. The amount of [^{125}I]IF_1 bound to complex V or soluble F_1-ATPase at maximal activity inhibition was estimated from titration curves to be 0.75–0.8 mol of [^{125}I]IF_1 per mole of complex V or F_1-ATPase. The conditions required for IF_1 to exert inhibition are incubation of IF_1 with an active ATPase at pH <7.0 in the presence of MgATP (or another hydrolyzable substrate). It was found that under otherwise optimal conditions, the alteration of any one of these factors (i.e., absence of MgATP, pH 8.0, or addition of rutamycin to inhibit ATPase activity) drastically diminished the binding of [^{125}I]IF_1 to the high-affinity site of complex V. It was shown by Galante et al. [Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1981) *Biochemistry 20*, 2671–2678] that the inhibition of complex V by IF_1 can be reversed by incubating the inhibited enzyme at pH > 7.0 in the absence of MgATP. The present studies have shown that reversal of inhibition is associated with the release of IF_1. In phosphate buffer at pH 8.0, the release of the radiolabeled inhibitor from [^{125}I]IF_1-treated complex V was found to precede the reappearance of ATPase activity, while in bicarbonate buffer of the same ionic strength and pH the reappearance of activity coincided with the release of [^{125}I]IF_1. An increase in ionic strength and the presence of other anions such as sulfite and nitrate, uncouplers, or Ca^{2+} did not duplicate the bicarbonate effect.

The bovine heart mitochondrial ATPase inhibitor protein (IF_1)[1] is a water-soluble, heat-stable polypeptide (Pullman & Monroy, 1963; Brooks & Senior, 1971). IF_1 consists of 84 amino acid residues with a molecular weight of 9578, and its sequence has been recently determined (Frangione et al., 1981). It inhibits ATP hydrolysis when incubated with F_1-ATPase, complex V (purified ATP synthetase complex), or submitochondrial particles at pH <7.0 in the presence of Mg^{2+}

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received May 10, 1982.* Supported by U.S. Public Health Service Grant AM 08126 and National Science Foundation Grant PCM 78-26790 to Y.H.

[1] Abbreviations: IF_1, ATPase inhibitor protein from bovine heart mitochondria; [^{125}I]IF_1, ^{125}I-labeled IF_1; F_1, soluble F_1-ATPase; F_0, membrane sector of the ATPase complex or complex V; NTP, nucleoside triphosphate; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; OSCP, oligomycin sensitivity conferring protein; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-5781$01.25/0

and a hydrolyzable nucleoside triphosphate (Pullman & Monroy, 1963; Racker & Horstman, 1967; Horstman & Racker, 1970; Gomez-Fernandez & Harris, 1978; Galante et al., 1981a,b). The requirement for MgATP was studied by Gomez-Fernandez & Harris (1978) with the conclusion that a large number of enzyme turnovers (200 or more) were necessary in order for the inhibitor to become effective. Galante et al. (1981a, 1982) studied the reversible inhibition of complex V ATPase activity by IF_1, confirmed the requirements for MgNTP and pH <7.0 to achieve inhibition, and showed in addition that incubation of IF_1-inhibited complex V, but not of IF_1-inhibited F_1-ATPase, at pH >7.0 and in the absence of MgNTP resulted in the reversal of inhibition. While the previous studies of others equated inhibition of ATPase activity with IF_1 binding, Galante et al. (1981a) pointed out that binding and release of IF_1 and correlation of such data with activity inhibition and reversal have yet to be demonstrated. Recently, however, Schwerzmann & Pedersen (1981) have shown in energized rat liver submitochondrial particles incubated at pH 7.6 that the rise in ATPase activity is accompanied by inhibitor protein release.

The present paper is concerned with the equilibrium binding of radiolabeled IF_1 to complex V under conditions favoring or not favoring inhibition of ATPase activity, as well as under conditions that activity inhibition is reversed. IF_1 was labeled with ^{125}I at the single tyrosyl residue of this polypeptide to yield an ^{125}I-labeled IF_1 with an inhibitor potency equal to that of native IF_1. Results have shown that IF_1 binds to complex V at high- and low-affinity sites. An active enzyme, the presence of MgNTP, and a pH <7.0 are required for IF_1 binding to the high-affinity site. The reappearance of ATPase activity at pH >7.0 coincides with or follows the release of IF_1 from complex V, depending on conditions.

Materials and Methods

Complex V was prepared from beef heart mitochondria and stored as described before (Stiggall et al., 1978, 1979). The preparation contained small and variable amounts of endogenous IF_1 which could cause up to 50% inhibition of ATPase activity under appropriate conditions, as shown previously (Galante et al., 1981a, 1982). Depletion of complex V preparations from endogenous IF_1 was accomplished as follows. Complex V, at a protein concentration of about 1 mg/mL, was suspended in 0.25 M sucrose containing 128 mM potassium bicarbonate, pH 8.2, and incubated at 30 °C for 30 min. As will be seen below, this treatment releases complex V bound IF_1. After centrifugation at 165000*g* for 60 min, the pellet was suspended in 0.25 M sucrose containing 50 mM Tris/acetate, pH 7.5, homogenized, and used immediately without freezing. This preparation will be referred to as IF_1-depleted complex V. The supernatant containing IF_1 was either discarded or concentrated by Cl_3CCOOH precipitation and assayed for IF_1 activity. IF_1 was purified essentially by the method of Horstman & Racker (1970) with minor modifications as described before (Galante et al., 1981a). The purity of IF_1 preparations was routinely checked by polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ on a 1.5-mm thick slab according to Laemmli (1970) as modified by Merle & Kadenbach (1980). F_1-ATPase was purified by the method of Senior & Brooks (1970) with the modifications described elsewhere (Galante et al., 1979). Before use, F_1-ATPase was desalted by the gel filtration/centrifugation method of Penefsky (1977) on Sephadex G-50 (fine) packed in a 1.0-mL tuberculin syringe and equilibrated in 0.25 M sucrose containing 50 mM Tris/acetate, pH 7.5. ATPase activity of complex V and F_1-ATPase was determined by the spectrophotometric method of Pullman et al. (1960) as described by Stiggall et al. (1978). IF_1 inhibition of ATPase activity was determined by the procedure of Horstman & Racker (1970) in 10 mM Tes/Tris or 10 mM potassium phosphate, pH 6.7, as described previously (Galante et al., 1981a) and specified in the figure legends.

Iodination of IF_1 was performed, using the procedure of Fraker & Speck (1979), on a solid phase composed of 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IODO-GEN) according to the recommendations of the Pierce Chemical Co. Fifty microliters of a 20 μg/mL freshly prepared solution of IODO-GEN dissolved in chloroform was added to each of a series of borosilicate disposable culture tubes (12 × 75 mm) and taken to dryness in a desiccator under reduced pressure. To each of 6–12 such "coated" tubes was added 100 μL of IF_1 at a concentration of 0.25–0.5 mg/mL in 5 mM Hepes/Tris, pH 7.4, followed by 1.0 μL of ^{125}I (40 mCi/mL). The tubes were incubated on ice for 15–20 min, the contents were pooled in a 1.7-mL Eppendorf tube, and KI was added to a final concentration of 2 mM. [^{125}I]IF_1 was precipitated with 5% Cl_3CCOOH and the precipitate collected by a 5-min centrifugation in an Eppendorf centrifuge. The pellet was resuspended in the same tube in about 300 μL of 50 mM Hepes/Tris, pH 7.4, and the Cl_3CCOOH precipitation step repeated 6–8 times until more than 95% of the counts remained associated with the pellet. Finally, [^{125}I]IF_1 was suspended in 50 mM Hepes/Tris, pH 7.4, and stored at −70 °C. The recovery of protein was virtually complete, and the incorporation of ^{125}I into IF_1 was between 10 and 15%. The specific radioactivity of [^{125}I]IF_1 was in the range of (1.8–4.5) × 10^8 cpm/mg.

Binding of [^{125}I]IF_1 to complex V or to IF_1-depleted complex V was measured as follows. The enzyme, at the protein concentrations indicated in the figure legends, was incubated with increasing amounts of [^{125}I]IF_1 at 30 °C for 20 min. Aliquots of 50 μL were withdrawn and centrifuged in a Beckman airfuge at 30 psi (roughly equivalent to 165000*g*) for 5 min. The pellet was resuspended in 50 μL of the same buffer used for the incubation and centrifuged as before. The protein content of the pellet (resuspended in 0.1 N NaOH) and of the combined supernatants was measured and the radioactivity determined in a Beckman 4000 γ counter. Protein concentration was measured by the biuret method of Gornall et al. (1949) in the presence of 1% potassium deoxycholate or by the method of Lowry et al. (1951).

IODO-GEN was from Pierce Chemical Co.; ^{125}I (carrier free) was obtained weekly from New England Nuclear with a radioactivity of 40 mCi/mL. Rutamycin was a gift from Eli Lilly & Co. The sources of all other chemicals were the same as before (Galante et al., 1981a, 1982).

Results

Radiolabeling of IF_1 with ^{125}I, under the condition described under Materials and Methods, did not affect its inhibitor activity. As shown in Figure 1, complex V was incubated in parallel experiments either with IF_1 or with [^{125}I]IF_1 and the ATPase activity measured after 20 min. The amounts of unlabeled and labeled inhibitor protein required for 50% inhibition were identical. By comparison, use of the chloramine-T radiolabeling procedure resulted in complete inactivation of IF_1 (not shown).

Figure 2 shows in the top trace (closed circles) the binding of [^{125}I]IF_1 to complex V as a function of increasing [^{125}I]IF_1 concentration. It is seen that the uptake of [^{125}I]IF_1 by complex V is biphasic and appears to consist of a saturable high-affinity phase followed by a low-affinity phase. The

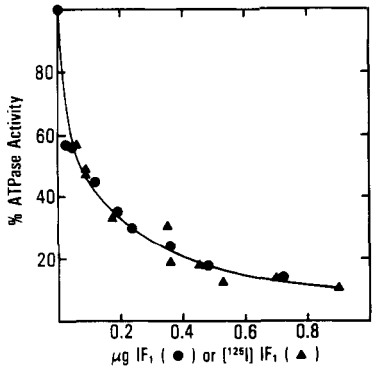

FIGURE 1: Inhibition of the ATPase activity of complex V by IF_1 or $[^{125}I]IF_1$. Complex V at a protein concentration of 1.2 mg/mL in a final volume of 0.1 mL of 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, was incubated at 30 °C for 20 min with 2 mM MgATP and with increasing amounts of IF_1 (●) or $[^{125}I]IF_1$ (▲) as shown. ATPase activity was measured as described under Materials and Methods. 100% ATPase activity of complex V was 6 μmol min^{-1} (mg of protein)$^{-1}$.

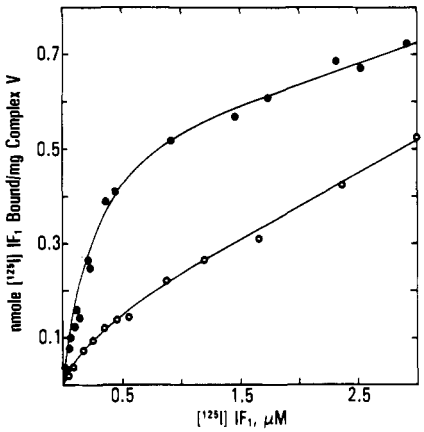

FIGURE 2: Equilibrium binding of $[^{125}I]IF_1$ to complex V and to complex V pretreated with IF_1. Complex V was incubated in two separate tubes under the same conditions as in Figure 1. To one tube (○) was added 7.5 μg of IF_1 per mg of protein, which after 20 min of incubation caused 80% inhibition of complex V ATPase activity. The second tube (●) received buffer equivalent to the volume of IF_1. Aliquots from each tube were further incubated with $[^{125}I]IF_1$ at the level of 0.5–25 μg of $[^{125}I]IF_1$ per mg of complex V or IF_1-treated complex V for 20 min at 30 °C in the presence of 2 mM MgATP. The amount of $[^{125}I]IF_1$ bound to complex V or IF_1-treated complex V was determined as described under Materials and Methods. 100% ATPase activity of complex V was 7.4 μmol min^{-1} (mg of protein)$^{-1}$.

second trace in Figure 2 (open circles) shows the result of an experiment in which the complex V used for $[^{125}I]IF_1$ binding was pretreated with unlabeled IF_1 such that 80% of its ATPase activity was inhibited. The use of incompletely inhibited complex V in this experiment was deliberate, since binding of the inhibitor protein to ATPase requires that the enzyme be active and capable of turning over (Gomez-Fernandez & Harris, 1978). That partially inhibited complex V is capable of IF_1 binding and maximal inhibition has been shown elsewhere (Galante et al., 1982). However, as seen in Figure 2, the results with IF_1-treated complex V showed that the high-affinity binding of $[^{125}I]IF_1$ was greatly diminished, and the labeled IF_1 binding was essentially low affinity in character. These results suggested, therefore, that complex V contains a saturable high-affinity site for IF_1 binding and that inhibition of ATPase activity appears to involve the occupation of this site by IF_1 (additional support for the latter conclusion is given below). That complex V should also contain low-affinity binding sites for IF_1 is not surprising. We have shown that IF_1 binds to a variety of substances, including complex III

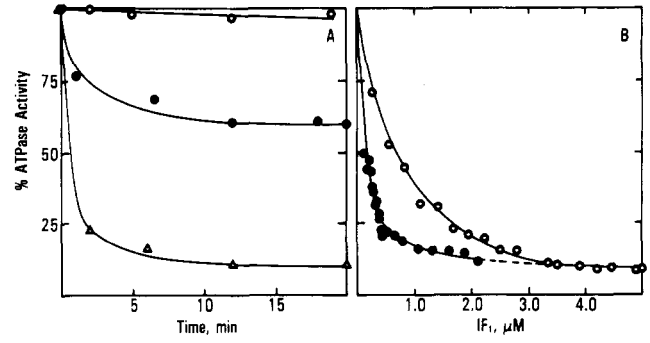

FIGURE 3: Comparison of the requirements for added IF_1 to inhibit complex V and IF_1-depleted complex V. (A) Effect of incubation with MgATP on complex V (●), IF_1-depleted complex V (○), and IF-depleted complex V supplemented with 15 μg of IF_1 per mg of IF_1-depleted complex V (△). (B) Titration of complex V (●) and IF_1-depleted complex V (○) with added IF_1. Buffer and incubation conditions were the same as in Figure 1. 100% ATPase activities of complex V and IF_1-depleted complex V were, respectively, 8 and 12 μmol min^{-1} (mg of protein)$^{-1}$.

(ubiquinol–cytochrome *c* oxidoreductase), microsomes, bovine serum albumin, glass, and cellulose nitrate centrifuge tubes.[2]

In a previous paper (Galante et al., 1981a), we showed that preparations of complex V contain variable amounts of IF_1, identifiable on two-dimensional $NaDodSO_4$–acrylamide gels of purified complex V and capable of exerting 25–50% inhibition when complex V was incubated at pH <7.0 with MgATP. This endogenous IF_1 of complex V is analogous to the IF_1 present in submitochondrial particles and chloroform-extracted F_1 (Lowe & Beechey, 1981a,b). For the present studies, it was desirable, however, to work with IF_1-free complex V. We had previously prepared complex V depleted of the inhibitor protein by reconstitution of IF_1-free urea–F_0 with purified F_1 and OSCP in the presence of phospholipids (Galante et al., 1981b). The reconstituted, IF_1-free complex V was unstable, however, and difficult to handle in binding experiments. Thus, the following procedure for the removal of IF_1 from preparations of complex V was worked out. Complex V was incubated at pH 8.0–8.2 in phosphate or bicarbonate buffer, which resulted in the release of endogenous IF_1 in the supernatant, and IF_1-depleted complex V was sedimented by ultracentrifugation. The following lines of evidence indicated that indeed IF_1 was separated from complex V by this simple manipulation: (a) The Cl_3CCOOH precipitate of the supernatant contained active IF_1. (b) The sedimented complex V showed no inhibition of ATPase activity when incubated at pH 6.7 in the presence of MgATP. (c) A higher concentration of added IF_1 was necessary in order to obtain 50% inhibition of ATPase activity with the depleted as compared to untreated complex V. Points b and c are illustrated in panels A and B, respectively, of Figure 3. In Figure 3A, complex V (closed circles) or IF_1-depleted complex V (open circles) was incubated at pH 6.7 in the presence of MgATP. The ATPase activity of complex V became about 40% inhibited in 10 min because of the presence of endogenous IF_1 (Galante et al., 1981a). By comparison, the IF_1-depleted complex V showed no inhibition up to 20 min of incubation. However, when IF_1 was added to the IF_1-depleted complex, then its ATPase activity (open triangles) was 90% inhibited under the same conditions of pH, temperature, and presence of MgATP. Figure 3B shows the inhibition of the ATPase activities of depleted and undepleted complex V by increasing

[2] The binding of IF_1 to containers and centrifuge tubes is not insignificant and should be taken into consideration in quantitative experiments.

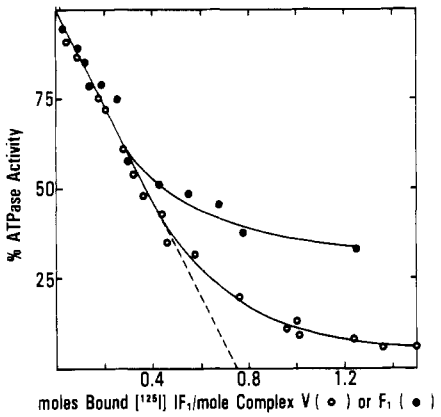

FIGURE 4: Titration with $[^{125}I]IF_1$ of activity and binding capacity of F_1-ATPase and IF_1-depleted complex V. Soluble F_1-ATPase (●) was dissolved at 0.5 mg/mL in 0.25 M sucrose containing 2 mM MgATP and 10 mM potassium phosphate, pH 6.7. IF_1-depleted complex V (○) was suspended at 1.0 mg/mL in the same medium, except that the buffer was 10 mM Mes/Tris, pH 6.7. Aliquots from each preparation were treated with the $[^{125}I]IF_1$ concentrations shown, incubated for 20 min at 30 °C, and assayed for ATPase activity and bound radioactivity. The radioactivity associated with IF_1-depleted complex V was determined as described under Materials and Methods. The radioactivity associated with F_1-ATPase was determined by using the Sephadex gel filtration method described by Klein et al. (1980). 100% ATPase activities of IF_1-depleted complex V and F_1-ATPase were, respectively, 14 and 70 μmol min^{-1} (mg of protein)$^{-1}$.

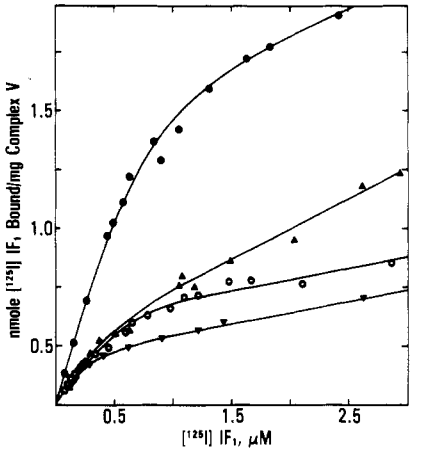

FIGURE 5: Effects of pH, MgATP, and rutamycin on the equilibrium binding of $[^{125}I]IF_1$ to IF_1-depleted complex V. IF_1-depleted complex V was suspended at 1.2 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, in the presence (●) or absence (○) of 2 mM MgATP, or in the presence of 2 mM MgATP and 10 μg of rutamycin per mg of protein (▼). In (▲), 2 mM MgATP was present, but the pH was 8.0. Aliquots from each tube were incubated for 20 min at 30 °C with the amounts of $[^{125}I]IF_1$ shown. Complex V bound and free $[^{125}I]IF_1$ were separated by centrifugation, and radioactivity associated with complex V was determined as described under Materials and Methods. 100% ATPase activity of IF_1-depleted complex V was 12 μmol min^{-1} (mg of protein)$^{-1}$.

amounts of added IF_1. It is seen that the IF_1-depleted complex V (open circles) required a 4-fold higher concentration of added IF_1 for 50% ATPase activity inhibition. A qualifying comment regarding the quantitative aspect of the IF_1 titers might be added. The depletion of IF_1 from complex V by the procedure described above also resulted in the removal of some bound cholate from the enzyme complex. Consequently, the suspension of the depleted complex V, even after thorough homogenization and brief sonication, was still cloudy, which might have affected the IF_1 titer, while the undepleted complex V readily dispersed in buffer, giving a clear solution (Stiggall et al., 1978).

A more precise way of studying the binding of IF_1 to IF_1-depleted complex V is shown in Figure 4. In this figure, the percent ATPase activity of IF_1-depleted complex V is plotted against the molar ratio of bound $[^{125}I]IF_1$ to complex V [assuming an M_r of 500 000; see also Berden & Voorn-Brouwer (1978)]. Extrapolation of the linear portion of the curve obtained (open circles) to zero ATPase activity indicated a ratio of 0.75–0.8 mol of $[^{125}I]IF_1$ bound per mol of complex V. For comparison, binding of $[^{125}I]IF_1$ to purified F_1-ATPase was also studied (Figure 4, closed circles). The same value of 0.75–0.8 mol of $[^{125}I]IF_1$ bound per mol of F_1 was found by a similar extrapolation to 0% activity. These results are in agreement with the values reported by Klein et al. (1980) for the binding of IF_1 derivatized with phenyl [^{14}C]isothiocyanate to soluble F_1-ATPase (i.e., 0.7–0.9 mol of ^{14}C-labeled IF_1 per mol of F_1). Thus, it may be concluded that maximal inhibition of the ATPase activities of soluble F_1-ATPase and the complete F_1–F_0 complex requires the binding of about 1 mol of IF_1 per mol of the enzyme. A similar stoichiometry has been reported for inhibition of F_1-ATPase by efrapeptin (Grubmeyer & Penefsky, 1981).

As mentioned above, inhibition by IF_1 of submitochondrial particles, soluble F_1-ATPase, and complex V requires the presence of an active enzyme, a hydrolyzable substrate, and Mg^{2+} and is promoted by pH <7.0 (Horstman & Racker, 1970; Gomez-Fernandez & Harris, 1978; Galante et al., 1981a). It was, therefore, of interest to see how these factors affect the high-affinity and low-affinity phases of IF_1 binding to complex V as shown in Figure 2. In Figure 2, the experiment was conducted with undepleted complex V, which contained some endogenous IF_1. In the experiments of Figure 5, IF_1-depleted complex V was used, which as seen in Figure 5 (top trace) resulted in a much more pronounced high-affinity binding phase. The other traces in Figure 5 show the effects of the above factors. Thus, incubation of IF_1-depleted complex V either at pH 8.0 in the presence of MgATP (closed upright triangles) or at pH 6.7 in the absence of MgATP (open circles) almost completely eliminated the high-affinity binding phase. The difference in the low-affinity binding phases of the two experiments might be, among other things, related to (a) the fact that in the presence of MgATP and pH 8.0 ATPase activity is still slowly inhibited by IF_1 (Galante et al., 1981a) and (b) differences in the electrostatic interaction of the proteins, even at the low-affinity sites, between pH 8.0 and pH 6.7. Particularly interesting, however, was the observation that incubation of $[^{125}I]IF_1$ with complex V plus MgATP, at pH 6.7, but in the presence of rutamycin to inhibit the ATPase activity by 90–95% (bottom trace, Figure 5), also inhibited the high-affinity binding phase. Thus, it is clear from Figure 5 that the factors necessary to effect inhibition of ATPase activity by IF_1, i.e., pH <7.0, the presence of MgATP, and the active state of the enzyme, are all conditions that promote IF_1 binding to the complex V high-affinity binding site. These results also show the relationship between IF_1 binding to the high-affinity site and inhibition of ATPase activity.

The data of Figure 5 also made it possible to analyze the IF_1 binding curve obtained under conditions favoring inhibition (i.e., top trace of Figure 5) and deduce the information relevant to the high-affinity binding of $[^{125}I]IF_1$. To do this, it was necessary to subtract the contribution due to low-affinity binding of $[^{125}I]IF_1$ from the overall binding curve represented by the top trace of Figure 5. The low-affinity binding contribution could be estimated from an experiment such as the top trace of Figure 5, provided additional binding data were also obtained for $[^{125}I]IF_1$ concentrations very much higher than those used. However, the use of very high concentrations

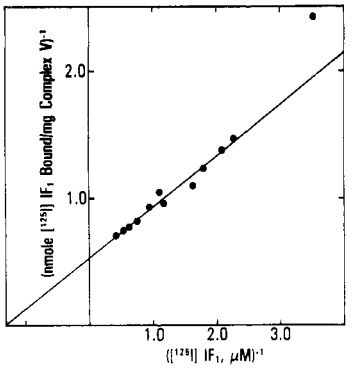

FIGURE 6: Double-reciprocal plot of the high-affinity binding of $[^{125}I]IF_1$ to IF_1-depleted complex V. The experimental data are those of Figure 5, top trace (●), from which an approximated low-affinity $[^{125}I]IF_1$ binding was subtracted. The latter was assumed to be a straight line passing through the origin and parallel to the straight-line segments of the experimental curves (▼) and (○) of Figure 5.

of $[^{125}I]IF_1$ was both impractical and technically problematic. Instead, we have assumed that the straight-line portion of the binding curve obtained with rutamycin-treated complex V (bottom trace of Figure 5) might be a close approximation of the low-affinity binding of $[^{125}I]IF_1$. The reason for selecting this curve was that, as in the top trace of Figure 5, the experiment with rutamycin-treated complex V was conducted at pH 6.7 and in the presence of MgATP. In addition, rutamycin, acting on the F_0 portion, would not be expected to alter potential binding sites on F_1. Thus, for the purpose at hand, it was assumed that the low-affinity binding of $[^{125}I]IF_1$ to complex V would give a straight line passing through the origin and parallel with the straight-line portion of the bottom trace of Figure 5 (note that the straight-line portion of the binding curve obtained at pH 6.7 in the absence of MgATP has the same slope). When this approximated low-affinity binding contribution was subtracted from the overall binding curve (top trace of Figure 5), a saturation curve was obtained, which gave a straight line in a double-reciprocal plot (Figure 6). The ordinate intercept of this line, representing the saturation level of the high-affinity binding site, indicated 1.88 nmol of $[^{125}I]IF_1$ bound per mg of complex V or 0.94 mol of the inhibitor per mol of complex V (M_r 500 000). This value is in excellent agreement with the data of Figure 4 and the results of Klein et al. (1980) discussed above. The abscissa intercept is a measure of the dissociation constant of $[^{125}I]IF_1$ with respect to the high-affinity binding site. This value, as calculated from Figure 6, was 0.75 μM.

In a previous paper (Galante et al., 1981a), we described the reversibility of IF_1-induced inhibition of complex V. The principal conditions that favor reversal of inhibition are pH >7.0 and absence of a hydrolyzable substrate. In other studies (Galante et al., 1982), the reversible inhibition of complex V by IF_1 was used to investigate whether the inhibitions brought about by IF_1 and various artificial inhibitors of F_1 were independent, mutually interfering, or mutually exclusive. In these studies, the fate of the bound IF_1 during reversal of inhibition could not be assessed. However, in the present work, the use of iodinated IF_1 allowed us to investigate the fate of $[^{125}I]IF_1$ during reactivation of the $[^{125}I]IF_1$-inhibited complex V and to answer the question of whether the inhibitor protein is released from the enzyme complex during reversal of inhibition or is simply displaced from its inhibitory site.

Figure 7 shows the time-dependent reversal of inhibition of ATPase activity and the release of the inhibitor from $[^{125}I]$-IF_1-inhibited complex V at pH 8.0 in phosphate and bicarbonate buffers of the same ionic strength. It is important

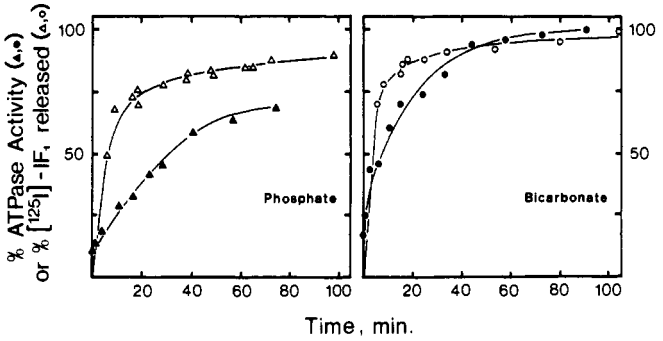

FIGURE 7: Reappearance of ATPase activity and release of $[^{125}I]IF_1$ as a function of incubation of $[^{125}I]IF_1$-inhibited complex V at pH 8.0 in phosphate or bicarbonate buffer. IF_1-depleted complex V was treated under the same conditions as described in Figure 1 with 7.0 μg of $[^{125}I]IF_1$ per mg of the complex. The mixture was centrifuged at 165000*g* for 1 h, and the pellet containing bound $[^{125}I]IF_1$ was resuspended at 1 mg/mL in 0.25 M sucrose containing either 50 mM potassium phosphate or 128 mM potassium bicarbonate at pH 8.0. Activity measurement indicated that 88% of the complex V ATPase activity had been inhibited by treatment with $[^{125}I]IF_1$. The two suspensions were then incubated at 30 °C and at the intervals shown sampled to assay for activity (▲, ●) and release of $[^{125}I]IF_1$ (△, ○). The latter was done by centrifuging 50-μL aliquots in the Beckman airfuge for 5 min and determining radioactivity in the supernatant and the pellet as described under Materials and Methods. The pellet radioactivity was determined after the supernatant was removed and the tubes were rinsed with buffer. The percent $[^{125}I]IF_1$ released was calculated on the basis of the fraction of total radioactivity (supernatant plus pellet) found in the supernatant. At 100% $[^{125}I]IF_1$ release, the radioactivity associated with the pellet was negligible. 100% ATPase activity was the activity of the starting IF_1-depleted complex V [15 μmol min^{-1} (mg of protein)$^{-1}$].

to point out that these results do not offer a precise time correlation between inhibitor release and reappearance of activity, because while the ATPase activity of any given aliquot withdrawn from the incubation mixture was estimated immediately, the release of $[^{125}I]IF_1$ could be quantitated only after a 5-min ultracentrifugation as described under Materials and Methods. Nevertheless, the data of Figure 7 suggest that in phosphate buffer the release of bound $[^{125}I]IF_1$ precedes the reversal of inhibition of ATPase activity, whereas in bicarbonate the two events are more or less coincidental, somewhat faster, and reach completion more quickly.

Discussion

It has been shown that IF_1 purified from bovine heart mitochondria can be labeled with ^{125}I at its single tyrosyl residue without any apparent modification of its inhibitor potency. The radiolabeled IF_1 so prepared was used to study its binding to complex V in the presence and absence of the factors which are required for IF_1 to exhibit inhibition of ATPase activity. When the uptake of $[^{125}I]IF_1$ by complex V was studied as a function of $[^{125}I]IF_1$ concentration under the conditions favoring inhibition, it was found that the inhibitor was taken up by complex V in a biphasic manner. At low $[^{125}I]IF_1$ concentrations, there was a large uptake of the inhibitor, which was followed by low and apparently linear uptake at higher $[^{125}I]IF_1$ concentrations. These results suggested the existence of high- and low-affinity IF_1 binding sites on complex V. While the latter might be unspecific binding of IF_1 as discussed above, the high-affinity binding site appeared to be a saturable site concerned with the regulatory effect of IF_1. Thus, subtraction of data approximating low-affinity binding from the overall binding curve resulted in a saturation-type curve, which in a double-reciprocal plot gave a straight line. Maximal IF_1 binding to the high-affinity site, as calculated from the ordinate intercept of the double-re-

ciprocal plot, was 0.94 mol of $[^{125}I]IF_1$ bound per mol of complex V, and the dissociation constant as calculated from the abscissa intercept was 0.75 μM. The activity titration data of Figure 4 for complex V and soluble F_1-ATPase indicated 0.75–0.8 mol of IF_1 bound at full inhibition per mol of either enzyme preparation. Similar results (0.7–0.9 mol of IF_1 per mol of F_1-ATPase) were reported by Klein et al. (1980) when they titrated the activity of soluble F_1 with the phenyl [^{14}C]isothiocyanate derivative of IF_1 and estimated the enzyme-bound radioactivity after Sephadex gel filtration. Thus, the data for both the water-soluble and membrane-associated forms of ATPase show that activity inhibition involves the binding of approximately 1 mol of IF_1 per mol of the enzyme. In addition, our results with complex V indicate that the ATPase contains a high-affinity binding site for IF_1 which is saturated by approximately 1 mol of IF_1 per mol of the enzyme. When complex V was first about 80% inhibited with native IF_1, the high-affinity binding of $[^{125}I]IF_1$ to this system was considerably reduced. Similarly, when any one of the conditions required for expression of IF_1 inhibition (pH <7.0, presence of MgATP, active state of the enzyme) was altered, again the high-affinity binding of IF_1 was greatly diminished. These results indicated, therefore, that (a) the high-affinity binding site on complex V is concerned with IF_1 inhibition of ATPase activity and (b) the conditions necessary for expression of IF_1 inhibition are also required for the binding of IF_1 to the high-affinity site.

With regard to the factors which are required for IF_1 binding to the ATPase high-affinity site and expression of inhibitor activity, it appears that MgNTP and the active state of the enzyme are required for the ATPase to acheive a conformation capable of IF_1 binding. This is because (a) rutamycin abolished IF_1 binding to the high-affinity site of complex V, even though it binds to F_0 and should not be expected to interfere directly with the F_1–IF_1 interaction, and (b) the rate and extent of inhibition achieved by IF_1 appear to be related to the rate at which MgNTP is hydrolyzed by the enzyme (Gomez-Fernandez & Harris, 1978; Galante et al., 1981a). However, the effect of pH appears to be concerned with the affinity between F_1 and IF_1 rather than with making the high-affinity site on F_1 available for IF_1 binding. The reason for this conclusion is that under otherwise appropriate conditions (i.e., active enzyme and presence of MgATP), IF_1 can inhibit complex V even at pH 8.0, which is optimal for inhibition reversal, and the inhibition can be reversed in the absence of MgATP even at pH 6.7, which in the presence of MgATP is the optimal pH for inhibition (Galante et al., 1981a). These considerations are summarized in eq 1 and 2

$$CV \underset{}{\overset{\text{MgNTP}}{\rightleftharpoons}} CV^* \quad (1)$$

$$CV^* + IF_1 \underset{\text{pH} > 7.0}{\overset{\text{pH} < 7.0}{\rightleftharpoons}} CV^*\text{–}IF_1 \quad (2)$$

where CV* is the conformational form of complex V capable of IF_1 binding at its high-affinity site. The fact that even at pH 8.0 IF_1 release and activity reappearance are inhibited when MgATP is present is explainable on the basis that MgATP will shift the equilibrium of eq 1 and 2 in the direction of CV*–CF_1 formation.[3]

The data of Figure 7, showing release of $[^{125}I]IF_1$ from complex V and reappearance of ATPase activity, seem to suggest that in phosphate buffer the reappearance of activity lags behind inhibitor release. The fact that measurement of $[^{125}I]IF_1$ release for the same activity assay time point involved an additional 5-min centrifugation (see Results) might be considered a complicating factor in comparing the degrees of inhibitor release and activity reappearance. However, when the same experiment was conducted in bicarbonate buffer (Figure 7), inhibitor release and activity reappearance appeared to be more or less coincidental, which weakens the argument for the artifactual nature of the differential obtained in phosphate. This conclusion is valid because the bicarbonate effect is not due to stimulation of ATPase activity. The 100% activity shown in Figure 7 is based on the activity of the IF_1-depleted complex V used in these experiments prior to addition of $[^{125}I]IF_1$, and this activity was unchanged when assayed in the presence of bicarbonate. Furthermore, the 100% release of $[^{125}I]IF_1$ means that the complex V pellet was essentially free of radioactivity. These considerations suggest, therefore, that in the absence of bicarbonate the release of IF_1 is not accompanied with the reappearance of activity and that after IF_1 release there might exist an intermediate, inactive form of ATPase whose conversion to active enzyme is accelerated by bicarbonate. It was shown by Ebel & Lardy (1975) that a number of anions, including bicarbonate, greatly stimulated the ATPase activity of rat liver mitochondria. With the most effective anions, the ATPase activity of submitochondrial particles was increased about 2-fold, while stimulation of soluble F_1-ATPase was slight. In addition, they showed that in the absence of stimulating ions Lineweaver–Burk plots of ATPase activity curved downward at high ATP concentrations and that this effect disappeared in the presence of bicarbonate. Similar results were reported by Pedersen (1976a,b), and both papers suggested that activating anions might bring about these changes by altering the conformation of the enzyme. More recently, Pedersen et al. (1981) have suggested that activating anions, such as bicarbonate, and the inhibitor protein might affect F_1 activity by acting on a common allosteric site. Whether these observations and conclusions are related to our results shown in Figure 7 remains to be seen. Ebel & Lardy (1975) showed that, among other things, sulfite and dinitrophenol also stimulated ATPase activity, but in our experiments, sulfite and dinitrophenol stimulated neither the release of $[^{125}I]IF_1$ from complex V nor the reappearance of ATPase activity. Furthermore, in experiments such as that shown in Figure 7, we observe no effect of Ca^{2+}, or carbonyl cyanide *m*-chlorophenylhydrazone. Yamada et al. (1981) have claimed that Ca^{2+} releases the inhibitor protein from rat skeletal muscle submitochondrial particles and dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone reverse the inhibition of ATPase activity by the purified inhibitor protein. It should also be added that the bicarbonate effect shown in Figure 7 is not due to ionic strength, since Tuena de Gomez-Puyou et al. (1980) have concluded that high ionic strength induces IF_1 detachment. The experiments of Figure 7 in phosphate and bicarbonate buffers were not only conducted at the same ionic strength but also the addition of salts to increase the ionic strength in phosphate buffer did not duplicate the bicarbonate effect.

[3] Equations 1 and 2 are consistent with data regarding added IF_1. They do not pertain to the endogenous IF_1 of complex V which is clearly tightly bound to the enzyme in a noninhibitory state but is capable of expressing inhibition when complex V is incubated at pH <7.0 in the presence of MgATP.

Acknowledgments

We thank C. Munoz for the preparation of mitochondria and complex V.

References

Berden, J. A., & Voorn-Brouwer, M. M. (1978) *Biochim. Biophys. Acta 501*, 424–439.

Brooks, J. C., & Senior, A. E. (1971) *Arch. Biochem. Biophys. 147*, 467–470.
Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 191–196.
Fraker, P. J., & Speck, J. C. (1979) *Biochem. Biophys. Res. Commun. 28*, 849–857.
Frangione, B., Rosenwasser, E., Penefsky, H. S., & Pullman, M. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7403–7407.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1979) *J. Biol. Chem. 254*, 12372–12378.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1981a) *Biochemistry 20*, 2671–2678.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1981b) *Arch. Biochem. Biophys. 211*, 643–651.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1982) *Biochemistry 21*, 680–687.
Gomez-Fernandez, J. C., & Harris, D. A. (1978) *Biochem. J. 176*, 967–975.
Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
Grubmeyer, C., & Penefsky, H. S. (1981) *J. Biol. Chem. 256*, 3718–3727.
Horstman, L. L., & Racker, E. (1970) *J. Biol. Chem. 254*, 1336–1344.
Klein, G., Satre, M., Dianoux, A.-C., & Vignais, P. V. (1980) *Biochemistry 19*, 2919–2925.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lowe, P. N., & Beechey, R. B. (1981a) *Biochem. J. 196*, 433–442.
Lowe, P. N., & Beechey, R. B. (1981b) *Biochem. J. 196*, 443–449.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Merle, P., & Kadenbach, B. (1980) *Eur. J. Biochem. 105*, 499–507.
Pedersen, P. L. (1976a) *J. Biol. Chem. 251*, 934–940.
Pedersen, P. L. (1976b) *Biochem. Biophys. Res. Commun. 71*, 1182–1188.
Pedersen, P. L., Schwerzmann, K., & Cintron, N. (1981) *Curr. Top. Bioenerg. 11*, 149–199.
Penefksy, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
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.
Racker, E., & Horstman, L. L. (1967) *J. Biol. Chem. 242*, 2547–2551.
Schwerzmann, K., & Pedersen, P. L. (1981) *Biochemistry 20*, 6305–6311.
Senior, A. E., & Brooks, J. C. (1970) *Arch. Biochem. Biophys. 140*, 257–266.
Stiggall, D. L., Galante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 956–964.
Stiggall, D. L., Galante, Y. M., & Hatefi, Y. (1979) *Methods Enzymol. 55*, 308–315.
Tuena de Gomez-Puyou, M., Nordenbrand, K., Muller, U., Gomez-Puyou, A., & Ernster, L. (1980) *Biochim. Biophys. Acta 592*, 385–395.
Yamada, E. W., Huzel, N. J., & Dickison, J. C. (1981) *J. Biol. Chem. 256*, 10203–10207.

Proteolipid of Adenosinetriphosphatase from Yeast Mitochondria Forms Proton-Selective Channels in Planar Lipid Bilayers†

Hansgeorg Schindler* and Nathan Nelson‡

ABSTRACT: Proteolipid isolated from yeast mitochondrial adenosinetriphosphatase by butanol extraction is reincorporated into lipid vesicles from which planar membranes are formed. The proteolipid permits electric conductance through the membrane. This conductance occurs through membrane channels which are highly selective for protons. Proton channels in the membrane are directly observed at high proton concentrations in the aqueous phases. Channels open and close independently from each other; their open-state conductances and lifetimes are monodisperse but influenced by the applied voltage (12 pS and 3 s, respectively, at pH 2.2 and 100 mV). Proton channels do not occur in single proteolipid molecules; the conducting structure consists of at least two polypeptide chains since channels form in a (reversible) bimolecular reaction of nonconducting forms of proteolipid. The number of proton channels at a constant proteolipid concentration changes in sharp transitions and by orders of magnitudes upon critical changes of membrane composition and pH. These transitions are caused by transitions of proteolipid organization in the membrane from a dispersed state (equilibrium between channel-forming "dimers" and a large pool of "monomers") to a state of almost complete aggregation of proteolipid which stabilizes large proton-conducting structures (probably associates of channel-forming dimers). This self-association of isolated proteolipid into structures containing proton-selective channels suggests that the six proteolipids in the adenosinetriphosphatase complex exist as a self-associating entity containing most likely three proton channels.

The *N,N'*-dicyclohexylcarbodiimide (DCCD)[1]-binding protein has been recognized as a main constituent of the proton channel in proton-translocating ATPases from bovine heart mitochondria (Racker, 1976, 1977), *Escherichia coli* membranes (Altendorf, 1977; Fillingame, 1980), and thermophilic bacteria (Kagawa, 1978). The first successful reconstitution was reported for DCCD-binding protein ("proteolipid") from

† From the Biocenter of the University of Basel, CH-4056 Basel, Switzerland. *Received May 7, 1982.* This work was supported by Swiss National Science Foundation Grant 3.390.78.
‡ Permanent address: Department of Biology, Technion, Haifa, Israel.

[1] Abbreviations: ATPase, adenosinetriphosphatase; DCCD, *N,N'*-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

0006-2960/82/0421-5787$01.25/0