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Uncoupling of Oxidative Phosphorylation: Different Effects of Lipophilic Weak Acids and Electrogenic Ionophores on the Kinetics of ATP Synthesis†

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ABSTRACT: Previous studies from this laboratory have shown that the kinetics of ATP synthesis by bovine heart submitochondrial particles (SMP) are modulated by the coupled rate of respiration between two extremes of V_{max} and apparent K_m's for ADP and P_i [Matsuno-Yagi, A., & Hatefi, Y. (1986) *J. Biol. Chem. 261*, 14031–14038; Hekman, C., Matsuno-Yagi, A., & Hatefi, Y. (1988) *Biochemistry 27*, 7559–7565]. Thus, with ADP as the variable substrate, ATP synthesis occurred with V_{max} = 200 nmol of ATP min^{-1} (mg of protein)$^{-1}$ at 30 °C and an apparent K_m^{ADP} = 2–4 μM at low rates of respiration, and with V_{max} = 11 000 nmol of ATP min^{-1} (mg of protein)$^{-1}$ at 30 °C and an apparent K_m^{ADP} = 120–160 μM at high rates of respiration. At intermediate respiration rates, it was necessary to introduce a third intermediate K_m^{ADP} for best fit of the kinetic data, indicating that transition from one kinetic extreme to the other is not abrupt and involves intermediate kinetic states of the ATP synthase complexes. The present paper shows that uncouplers affect the kinetics of ATP synthesis by SMP in two ways. When used at moderate concentrations, electrogenic ionophores such as gramicidin D or valinomycin plus nigericin decreased the V_{max} for ATP synthesis without changing the contributions of the low, intermediate, and high K_m^{ADP} to the overall rate of ATP synthesis. By contrast, potent lipophilic weak acid uncouplers, such as FCCP, CCCP, S-13, and SF6847, decreased V_{max} and converted the kinetics of ATP synthesis toward high K_m^{ADP}. Similar results were obtained when P_i was the variable substrate, or when the energy-linked reaction studied was ATP-driven reverse electron transfer from succinate to NAD, with NAD as the variable substrate. When the ATP synthase complexes of SMP were fractionally inactivated by dicyclohexylcarbodiimide, and as a result the kinetics of ATP synthesis by these particles were converted to the high-K_m mode, then partial uncoupling of oxidative phosphorylation by FCCP resulted in large increases in the apparent K_m for ADP and P_i. These results have been interpreted as follows. In the absence of uncouplers, increases in the apparent K_m^{ADP} and $K_m^{P_i}$ are associated with increased rates of coupled respiration and increased rates of proton flux through the ATP synthase complexes. Lipophilic weak acid uncouplers, but not gramicidin D and valinomycin plus nigericin when used at moderate uncoupling concentrations, react with the ATP synthase complexes and increase slippage in the coupling mechanism within the enzyme complex. As a result, uncoupled proton flux through the ATP synthase complex increases and results in increased apparent K_m values for ADP and P_i even though the rate of ATP synthesis decreases. A similar interpretation applies to the uncoupler-induced increase in the apparent K_m^{NAD} during ATP-driven reverse electron transfer from succinate to NAD. This interpretation is also consistent with the very high apparent K_m^{ADP} and $K_m^{P_i}$ obtained when SMP containing fractionally inactivated ATP synthases were partially uncoupled by FCCP. In these SMP preparations, the remaining, active ATP synthase complexes turn over very rapidly during oxidative phosphorylation [Matsuno-Yagi, A., & Hatefi, Y. (1988) *Biochemistry 27*, 335–340]. Partial uncoupling by a lipophilic weak acid, such as FCCP, further increases the proton flux through these active ATP synthases via the slip mechanism, thus resulting in very high apparent K_m values for ADP and P_i.

The mechanism of protonic energy transfer in oxidative and photosynthetic phosphorylation has remained a matter of debate since the advent of the chemiosmotic hypothesis in 1966 (Mitchell, 1966). The fact that energy coupling can take place in accordance with the chemiosmotic hypothesis via bulk to bulk $\Delta\tilde{\mu}_{H^+}$[1] has been amply demonstrated [see, for example, Racker and Stoeckenius (1974), Thayer and Hinkle (1975a,b),

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[1] Abbreviations: SMP, submitochondrial particle(s); $\Delta\psi$, membrane potential; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential of protons; F_oF_1, ATP synthase complex; F_o, membrane sector of F_oF_1; CF_o–CF_1, chloroplast F_oF_1; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; S-13, 2,5-dichloro-3-*tert*-butyl-4'-nitrosalicylanilide; SF6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)-malononitrile; I_{50}, uncoupler concentration required for 50% uncoupling.

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Boyer et al. (1977), Takabe and Hammes (1981), and Van Der Bend et al. (1984)]. At the same time, however, the lack of a correlation between changes in the energy input and outflow rates and the magnitude of steady-state $\Delta\tilde{\mu}_{H^+}$ has lent increasing support to views that protonic energy transfer takes place mainly within the membrane or at the membrane–water interface (Williams, 1978; Kell, 1979; Ferguson & Sorgato, 1981; Westerhoff et al., 1984a,b; Ferguson, 1985; Rottenberg, 1985; Slater et al., 1985, Beard & Dilley, 1986; Slater, 1987; Kamp et al., 1988). In chloroplasts especially, the studies of Dilley and others have suggested the existence of an intramembranal proton pool originating at photosystem II and capable of driving ATP synthesis by CF_o–CF_1 (Theg & Homann, 1982; Theg & Junge, 1983; Dilley & Schreiber, 1984; Prochaska & Dilley, 1987a,b).

One of the powerful approaches for probing the path of energy transfer in mitochondria, chloroplasts, and bacterial membranes has been the study of the mechanism of uncoupling. A large number of potent uncouplers, such as the lipophilic weak acid uncouplers, have been shown to increase membrane proton conductance and collapse $\Delta\tilde{\mu}_{H^+}$, in complete agreement with the chemiosmotic explanation for the mechanism of uncoupling (Mitchell, 1966, 1979; Terada, 1981; Nichols, 1982; Kasianowicz et al., 1987). Ionophores such as valinomycin and nigericin (in the presence of K^+) or gramicidin (in the presence of Na^+) also appear to uncouple via the same principle, i.e., collapse of $\Delta\tilde{\mu}_{H^+}$. On the other hand, anesthetics (Rottenberg, 1983) and fatty acids [Rottenberg & Steiner-Mordoch, 1986; Rottenberg & Hashimoto, 1986; see, however, Luvisetto et al. (1987) and Pietrobon et al. (1987)] are considered to uncouple by a different mechanism, since these reagents suppress $\Delta\tilde{\mu}_{H^+}$ much less than the rate of ATP synthesis or reverse electron transfer. Therefore, anesthetics and fatty acids are considered by Rottenberg (Rottenberg, 1983, 1985; Rottenberg & Hashimoto, 1986) to uncouple by interfering with an intramembranal pathway of energy transfer at specific sites. Other reagents that have been shown to uncouple by interacting at specific membrane sites are modifiers of mono- and dithiols (Yagi & Hatefi, 1984). These compounds modify sulfhydryl groups in F_o of the ATPase complex and cause reversible (except when alkylating agents are used) uncoupling of mitochondrial energy-linked reactions and collapse of $\Delta\tilde{\mu}_{H^+}$.

In the course of studies on the kinetic modalities of oxidative phosphorylation (Matsuno-Yagi & Hatefi, 1986; Hekman et al., 1988), we found that, at partially uncoupling concentrations, certain uncouplers of the lipophilic weak acid variety changed the kinetics of ATP synthesis in the direction of high K_m for ADP and P_i [see also Kayalar et al. (1976) and Hatefi et al. (1982)], while others (e.g., gramicidin D or valinomycin plus nigericin when used at moderate uncoupling concentrations) caused uncoupling without altering the contributions of the low- and high-K_m modes to the overall kinetics of oxidative phosphorylation. These findings, which will be described below, are of considerable interest. They demonstrate differences in the mode of action of various uncouplers that study of the mechanism of uncoupling with the use of artificial lipid membranes cannot possibly reveal. They also show a new feature of the lipophilic weak acid uncouplers that may involve local effects at the level of the energy-transducing enzyme complexes of the oxidative phosphorylation system.

Materials and Methods

Preparations and Assays. SMP were prepared from bovine heart mitochondria as reported elsewhere (Matsuno-Yagi & Hatefi, 1985). Protein concentration was determined by the method of Lowry et al. (1951). Oxidative phosphorylation activity was measured essentially as described before (Matsuno-Yagi & Hatefi, 1985, 1987). In a final volume of 0.6 mL, the reaction mixtures at pH 7.5 contained 0.25 M sucrose, 50 mM Tris–acetate, 0.5 mM EDTA, 25 mM glucose, 5 mM $MgCl_2$, 20 mM potassium phosphate containing (5–15) × 10^5 cpm of ^{32}P, 1–1200 μM ADP, 70 μg of hexokinase/mL, and 50 μg of SMP/mL (or as otherwise stated). When NADH was the respiratory substrate, the mixture was preincubated for 5 min at 30 °C; then the reaction was initiated by the addition of 0.5 mM NADH. When succinate was the oxidizable substrate, 6.7 mM potassium succinate (pH 7.5) was included during preincubation (in the absence of ADP) to activate succinate dehydrogenase and ensure linearity of oxygen uptake with time. Then, oxidative phosphorylation was initiated by the addition of ADP. The reaction was terminated after 4 min by the addition of 60 μL of 35% perchloric acid. Precipitated protein was removed by centrifugation for 5 min at top speed in a clinical centrifuge, and 0.5 mL of the supernatant was used for estimation of esterified ^{32}P essentially according to Pullman (1967) as described by Stiggall et al. (1979). Regardless of the nature of the respiratory substrate and uncouplers used in the assay and the ADP concentration employed, ^{32}P esterification was linear with time within the reaction time employed (4 min unless otherwise indicated). ATPase (Matsuno-Yagi & Hatefi, 1984) and electron transfer (Matsuno-Yagi & Hatefi, 1986) activities of SMP were measured as in the references given. ATP-driven reverse electron transfer from succinate to NAD was assayed as before (Hekman et al., 1988), except that the ATP concentration was 2 mM. Uncouplers were all dissolved in ethanol and added directly to the reaction mixtures. Ethanol concentration in the reaction mixture never exceeded 0.5%, which had no effect on the measured activities. It was also shown that in the concentration range employed, none of the uncouplers had any inhibitory effect on the rate of succinate or NADH oxidation by SMP or on the coupled enzymic systems used for the assays. The SMP preparations used showed under oxidative phosphorylation assay conditions succinate and NADH oxidation rates, respectively, of 800–850 and 1500–1700 nmol min^{-1} (mg of protein)$^{-1}$. All the activities were measured at 30 °C.

Treatment of SMP with Inhibitors. SMP at 10 mg/mL were incubated on ice with DCCD in a buffer containing 0.25 M sucrose and 50 mM Tris–acetate, pH 7.5. The DCCD concentrations and the incubation times are given in the figure legends. The SMP samples were then passed through two successive Sephadex centrifuge columns (G-50 fine) to remove unbound inhibitor and then assayed for oxidative phosphorylation activity. Control SMP were treated as above in the absence of DCCD. Under the conditions employed, treatment of SMP with DCCD had no effect on the uncoupled rates of NADH or succinate oxidation. When tributyltin chloride was used as the ATPase inhibitor, SMP were incubated with the inhibitor (up to 20 nM) for 1 h on ice.

Materials. FCCP and gramicidin D were obtained from Sigma. Valinomycin and CCCP were from CalBiochem. Nigericin was from Robert L. Hamill (Eli Lilly). SF6847 was a gift of Dr. H. Terada, Tokushima University (Japan). S-13 was from Monsanto. The sources of all other chemicals were as indicated previously (Matsuno-Yagi & Hatefi, 1985, 1986, 1987).

Results

Uncoupler Titer and SMP Concentration in the Assay Medium. A survey of the literature did not allow us to determine whether the effective concentrations of the various

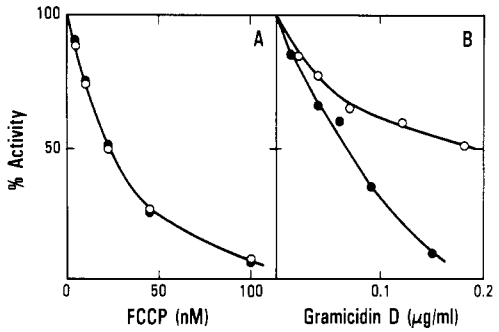

FIGURE 1: Effectiveness of FCCP and gramicidin D in uncoupling oxidative phosphorylation at different SMP concentrations. The rate of ATP synthesis was measured with succinate as the respiratory substrate as described under Materials and Methods. Uncouplers were added to the reaction mixtures as indicated. SMP concentrations were 0.062 (○) and 0.021 (●) mg/mL in panel A and 0.06 (○) and 0.02 (●) mg/mL in panel B. 100% activities in panels A and B were 817 and 808 nmol of ATP formed min^{-1} (mg of protein)$^{-1}$, respectively.

uncouplers we planned to use in this study were or were not dependent on enzyme (SMP) concentration in the assay medium. Our own experiments indicated that there was no general and easily rationalizable rule either. The effective concentrations of several uncouplers, including FCCP, CCCP, SF6847,[2] and nigericin (in the presence of fixed amounts of valinomycin and K^+), were independent of the SMP concentration in the assay. By contrast, the effective uncoupling concentration of gramicidin D was influenced by the SMP concentration in the assay. Figure 1 shows an example of the results with FCCP and gramicidin D. The effective uncoupling concentrations of S-13 and valinomycin (in the presence of fixed amounts of nigericin and K^+) were also influenced by the SMP concentration, but to a much smaller extent than that shown for gramicidin D in Figure 1B. In view of these results, it was decided to keep the concentration and total amount of SMP constant in the relevant experiments. Furthermore, only two SMP preparations were used with comparable activities for NADH and succinate oxidation and ATP synthesis.

Kinetics of Oxidative Phosphorylation Partially Uncoupled with Various Uncouplers. We have shown elsewhere that the kinetics of ATP synthesis by bovine heart SMP are modulated by the rate of energy production by the respiratory chain between two extremes. At low rates of coupled respiration, ATP synthesis occurs with low apparent K_m's for ADP and P_i and a low V_{max}. At high rates of coupled respiration, both the apparent K_m values and the V_{max} for ATP synthesis increase considerably. For example, with ADP as the variable substrate, apparent K_m^{ADP} increased by about 50-fold from 2–4 to 120–160 μM, and V_{max} increased similarly from about 200 nmol of ATP min^{-1} (mg of SMP protein)$^{-1}$ to about 11 000 nmol of ATP min^{-1} (mg of SMP protein)$^{-1}$. The transition from the low-K_m to the high-K_m mode was not abrupt. At intermediate rates of coupled respiration, the kinetic data required introduction of at least a third apparent K_m^{ADP}, suggesting the involvement of intermediate catalytic states of the ATP synthase complex between the two extremes described.

Since the rate of coupled respiration appeared in these studies to be the modulator of the kinetics of ATP synthesis by SMP, it was of interest to see what would happen to these kinetic modes when, instead of attenuating the rate of respiration, the system was partially uncoupled. One might have

[2] Muraoka et al. (1975) have reported that the I_{50} for SF6847 increased with increasing enzyme concentration. However, these investigators used whole mitochondria, not SMP, and they assayed for ATP–[^{32}P]P_i exchange activity.

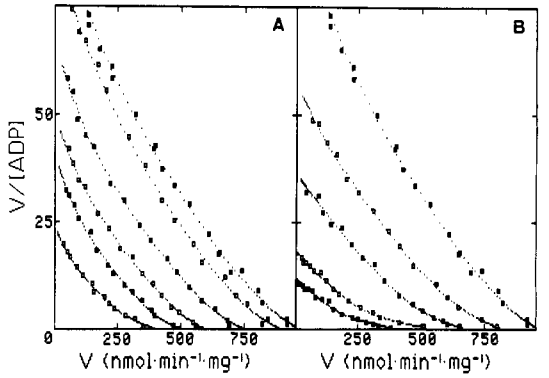

FIGURE 2: Effect of increasing concentrations of uncouplers on the kinetics of ATP synthesis. Conditions for assay of oxidative phosphorylation in the presence of variable ADP concentrations (0.9–1100 μM) were as given under Materials and Methods. The respiratory substrate was succinate. In panel A, gramicidin D was added to the reaction mixtures at a concentration (from top to bottom) of 0, 0.01, 0.036, 0.072, 0.12, and 0.25 μg/mL. In panel B, FCCP was added to the reaction mixtures at a concentration (from top to bottom) of 0, 7.5, 15, 30, and 42 nM. V, nanomoles of ATP formed per minute per milligram of protein. This and Figures 3–5 are computer printouts. The dots represent the curves calculated from the K_m and V_{max} values shown in Tables I–III.

expected that partial uncoupling would favor conversion of high-K_m–high-V_{max} kinetics to low-K_m–low-V_{max} kinetics. However, this did not happen, nor did all uncouplers exhibit the same effect on the kinetics of ATP synthesis. Two examples are shown in Figure 2A,B, which are Eadie–Hofstee plots ($v/[S]$ versus v) of the kinetics of ATP synthesis with ADP as the variable substrate. In panel A, the uncoupler was gramicidin D, and it is seen that increasing concentrations of gramicidin D lowered the V_{max} (abscissa intercept) from about 950 nmol of ATP min^{-1} (mg of SMP protein)$^{-1}$ to less than 400 nmol of ATP min^{-1} (mg of protein)$^{-1}$, but the shapes of the curves (i.e., the contributions of the low, intermediate, and high K_m^{ADP} to the overall kinetics of ATP synthesis) remained essentially unchanged. Similar results were obtained when uncoupling was achieved with moderate uncoupling concentrations of valinomycin plus nigericin. Progressive uncoupling with increasing levels of nigericin plus a fixed amount of valinomycin, or increasing levels of valinomycin plus a fixed amount of nigericin gave essentially similar results: V_{max} was decreased, but the curvilinear plots of the kinetics of ATP synthesis under partially uncoupled conditions were essentially parallel to the control (data not shown).

More contrary to expectation were the results shown in Figure 2B. Here the uncoupler was FCCP, and it is seen that progressive uncoupling was associated with a change in the kinetics of ATP synthesis toward high K_m^{ADP}. The difference between the effects of gramicidin D and FCCP on the kinetics of ATP synthesis is highlighted in Figure 3A, in which the concentrations of the two uncouplers are such that the degree of uncoupling as judged from the lowering of V_{max} is the same in the two experiments. However, as the two plots clearly show, the contribution to the overall rate of ATP synthesis is mainly from the low- and intermediate-K_m^{ADP} modes in the upper curve in which gramicidin D is the uncoupler, and from the high-K_m^{ADP} mode in the lower curve in which FCCP is the uncoupler. Figure 3B shows the results of an experiment with the same two uncouplers and P_i, rather than ADP, as the variable substrate. The data of Figure 3B demonstrate that the kinetic modality change demonstrated in Figures 2 and 3A with respect to K_m^{ADP} is applicable to $K_m^{P_i}$ as well. Results similar to those with FCCP as the uncoupler were also obtained with CCCP, S-13, and SF6847 (data not shown). At

Table I: Analysis of the Kinetics of ATP Synthesis in the Presence of Uncouplers with ADP or P_i as the Variable Substrate[a]

uncoupler	K_{m1}	V_1	% V_t	K_{m2}	V_2	% V_t	K_{m3}	V_3	% V_t	V_t
Variable Substrate = ADP										
none	2.7	110	11	13.2	716	74	154	142	15	968
gramicidin D (μg/mL)										
0.01	2.6	101	11	13.0	631	71	146	151	17	883
0.036	2.7	83	11	15.2	537	72	143	121	16	741
0.072	2.7	56	10	15.2	440	75	142	91	16	587
0.12	2.4	43	9	15.2	350	71	157	99	20	492
0.25	2.0	15	4	15.2	248	62	155	135	34	398
valinomycin (50 ng/mL) plus										
none	2.1	124	14	14.9	656	74	143	103	12	883
nigericin, 5 ng/mL	2.1	90	14	15.1	461	74	142	74	12	625
nigericin, 12.5 ng/mL	2.2	29	9	17.2	235	72	163	64	19	328
nigericin (50 ng/mL) plus										
none	2.5	100	10	16.8	711	73	150	166	17	977
valinomycin, 3 ng/mL	2.6	69	10	15.1	481	74	146	103	16	653
valinomycin, 9 ng/mL	2.1	32	10	15.1	214	69	158	63	20	309
FCCP (nM)										
7.5	2.4	30	4	13.1	581	71	154	208	25	819
15	3.6	5	1	13.0	438	65	156	231	34	674
30	3.8	4	1	14.9	231	44	160	296	56	531
42	–	–	–	15.8	165	42	152	229	58	394
CCCP (0.1 μM)	2.5	13	2	15.5	193	34	138	356	63	562
SF6847 (8.3 nM)	2.6	10	2	14.0	138	30	151	318	68	466
S-13 (3 nM)	2.2	4	1	13.6	228	39	166	356	60	588
Variable Substrate = P_i										
none	0.16	526	57	2.36	397	43				923
gramicidin D, 0.062 μg/mL	0.17	284	59	2.11	196	41				480
FCCP, 30 nM	0.19	65	14	2.18	413	86				478

[a] The K_m and V_{max} (V_1, V_2, V_3) values were derived from computer analysis of Eadie–Hofstee plots. The variable substrate was ADP (0.9–1100 μM) in the upper part and P_i (0.12–20 mM) in the lower part. The data for gramicidin D and FCCP as the uncouplers were derived from the Eadie–Hofstee plots of Figure 2. Assay conditions are given under Materials and Methods. In the bottom section, the data were analyzed by using two kinetic components. Dashes denote negligible contributions from those components in the data sets analyzed. The units for K_m and V_{max} in this and subsequent tables are micromolar and nanomoles of ATP formed per minute per milligram of protein, respectively. V_t is the sum of V_{max} values $V_1 + V_2 + V_3$.

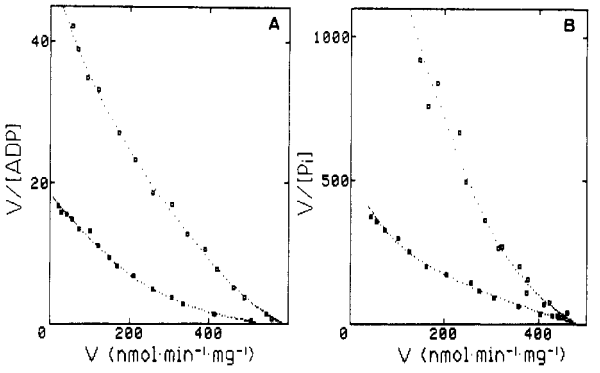

FIGURE 3: Effects of gramicidin D and FCCP at comparable uncoupling concentrations on the kinetics of ATP synthesis with ADP (panel A) and P_i (panel B) as the variable substrate. Assay procedures were the same as in Figure 2. Panel A is a replot of the curves for gramicidin D (□, top curve) (0.072 μg/mL in Figure 2A) and FCCP (■, bottom curve) (30 nM in Figure 2B). In panel B, assays were carried out as in (A), except that the variable substrate was P_i (0.12–20 mM). The reaction mixture in panel B contained 0.062 μg/mL gramicidin D (□, top curve) or 30 nM FCCP (■, bottom curve). V, nanomoles of ATP formed per minute per milligram of protein.

partially uncoupling concentrations, these reagents also converted the kinetics of ATP synthesis toward the high-K_m^{ADP} mode.

Analyses of the kinetic data for all the uncouplers mentioned above are summarized in Table I. As before, the data with ADP as the variable substrate were analyzed in all cases for three K_m^{ADP} values. The columns showing V_1, V_2, and V_3 relative to total V_{max} (% V_t) demonstrate the effects of the various uncouplers. It is seen that with gramicidin D as the uncoupler, the contributions of V_1, V_2, and V_3 to total V_{max} remained unchanged up to about 50% uncoupling (at higher gramicidin D concentrations, the contribution of V_1 decreased and that of V_3 increased).[3] The results with valinomycin and nigericin were essentially the same, in that partial uncoupling did not change the contributions of V_1, V_2, and V_3 to total V_{max}. However, with FCCP, CCCP, SF6847, and S-13, the contribution of V_1 to total V_{max} diminished sharply and that of V_2 also decreased, while the contribution of V_3 (the high-K_m component) increased by 4-fold as the SMP were 50 ± 10% uncoupled with FCCP, CCCP, S-13, or SF6847. The lower part of Table I shows the analysis of the data of Figure 3B in which P_i was the variable substrate. Other studies showed that the differences documented in Table I between ionophores and lipophilic weak acids were not confined to the kinetics of ATP synthesis. In ATP-driven reverse electron transfer from succinate to NAD, similar results were also obtained. Partial uncoupling by gramicidin D reduced V_{max} without altering the apparent K_m for NAD, while reduction of V_{max} by FCCP was accompanied by an increase in K_m^{NAD} (data not shown). The above results suggest, therefore, that, although ionophores and lipophilic weak acids may have comparable effects on $\Delta\tilde{\mu}_{H^+}$, there may be differences in the way these two groups of uncouplers interact with the mitochondrial inner membrane. They also underscore the known fact that diminution of steady-state $\Delta\tilde{\mu}_{H^+}$ by partial uncoupling has a very different effect on the kinetics of mitochondrial energy-driven reactions as compared to when $\Delta\tilde{\mu}_{H^+}$ is depressed by attenuation of the rate of energy production [see, for example, Zoratti et al. (1982)].

Effect of Partial Uncoupling on the Kinetics of ATP Synthesis When the Rate of Energy Production Relative to the

[3] Because of possible complications, high concentrations of ionophores were avoided in these studies.

Table II: Analysis of the Kinetics of ATP Synthesis in the Presence of FCCP as the Uncoupler and NADH as the Respiratory Substrate[a]

[FCCP] (nM)	K_{m1}	V_1	% V_t	K_{m2}	V_2	% V_t	K_{m3}	V_3	% V_t	V_t
0	3.1	62	2	14	732	27	146	1896	71	2690
45	3.7	27	1	19	297	17	203	1478	82	1802
94	3.7	21	2	21	115	9	280	1172	89	1308
171	4.2	7	1	24	31	6	323	497	93	535

[a] The K_m and V_{max} (V_1, V_2, V_3) values were derived from computer analysis of the Eadie–Hofstee plots of Figure 4.

Table III: Analysis of the Kinetics of ATP Synthesis Catalyzed by SMP Containing 22% Active F_oF_1 Complexes As Affected by Partially Uncoupling Concentrations of FCCP[a]

[FCCP] (nM)	K_{m1}	V_1	% V_t	K_{m2}	V_2	% V_t	V_t
0	15	39	3	152	1224	97	1263
50	23	37	5	237	681	95	718
100	44	25	4	345	550	96	575
200	98	11	3	802	326	97	337

[a] The respiratory substrate was NADH. The K_m and V_{max} (V_1, V_2, V_3) values were derived from computer analysis of the Eadie–Hofstee plots of Figure 5.

Number of Functional ATP Synthase Complexes Is Very High. The experiments described in the preceding section were conducted with succinate as the respiratory substrate. Under phosphorylating conditions, the SMP preparations used oxidized succinate at a rate of 800–850 nmol min^{-1} (mg of protein)$^{-1}$ and catalyzed ATP synthesis at a V_{max} rate of about 1000 nmol min^{-1} (mg of protein)$^{-1}$. The results, as seen in Table I, could be analyzed with the use of the same set of K_m^{ADP} and $K_m^{P_i}$ values that were derived previously (Hekman et al., 1988). Thus, with ADP as the variable substrate, K_m^{ADP} values of 2–4, 13–17, and 120–160 μM satisfied all the kinetic data (Table I). However, when NADH was used as the respiratory substrate, it was found that the K_m^{ADP} change induced by the lipophilic weak acid uncouplers exceeded the high-K_m^{ADP} range indicated above. Thus, as seen in Figure 4 and Table II, the smallest high K_m^{ADP} needed to satisfy the kinetic data at 80% uncoupling by FCCP was 323 μM. This increase in high K_m^{ADP} was presumably caused by the high rate of energy production by NADH, which was oxidized at a rate of about 1600 nmol min^{-1} (mg of protein)$^{-1}$, and suggested that under such conditions (i.e., partial uncoupling by lipophilic weak acids and high rate of energy production) the upper limit of 120–160 μM for K_m^{ADP} found previously no longer holds.

These previous studies had shown the following. (i) At high rates of coupled respiration relative to the number of functional ATP synthase complexes, the kinetics of ATP synthesis shifted toward the high-K_m mode for ADP and P_i (Matsuno-Yagi & Hatefi, 1986). (ii) Fractional inactivation of the ATP synthase complexes increased the turnover rate for ATP synthesis of the remaining, active F_oF_1 complexes to a V_{max} of 440 s^{-1} (11 000 nmol of ATP min^{-1} mg^{-1}) with either succinate or NADH as the respiratory substrate (Matsuno-Yagi & Hatefi, 1988). (iii) Under these conditions of high turnover rates of the F_oF_1 complexes, the kinetics of ATP synthesis became essentially monophasic with a high K_m^{ADP} of 120–160 μM (Hekman et al., 1988). However, as seen in Figure 4 and Table II, it seemed that, in the presence of lipophilic weak acid uncouplers, this upper limit for K_m^{ADP} can be exceeded when NADH is used as the energy source and the rate of respiration is high. Thus, it was of interest to see how high this apparent K_m^{ADP} can go when the rate of energy production relative to the number of functional ATP synthases is further increased by fractional inactivation of the F_oF_1 complexes. As before, the F_oF_1 complexes of SMP were fractionally and irreversibly inactivated with DCCD under conditions (0 °C, neutral pH) that DCCD had been shown to react in SMP only with the proteolipid subunit of F_o (Matsuno-Yagi & Hatefi, 1988). These SMP preparations were then used to catalyze ATP synthesis, with NADH as the energy source, ADP as the variable substrate, and FCCP as the uncoupler. Results are shown in the Eadie–Hofstee plots of Figure 5 and analyzed in Table III. It is seen that under these conditions, the apparent high K_m^{ADP} greatly exceeded the 120–160 μM limit found in the absence of uncouplers. Indeed, the data of Table

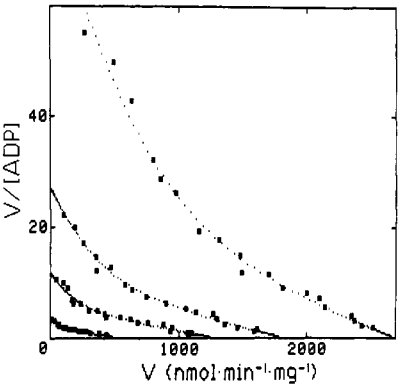

FIGURE 4: Effect of FCCP on the kinetics of ATP synthesis driven by NADH oxidation. The variable substrate was ADP (5–2000 μM). FCCP was added to the reaction mixtures at a concentration (from top to bottom) of 0, 45, 94, and 171 nM. Other conditions were the same as described under Materials and Methods, except that the reaction time was 195 s.

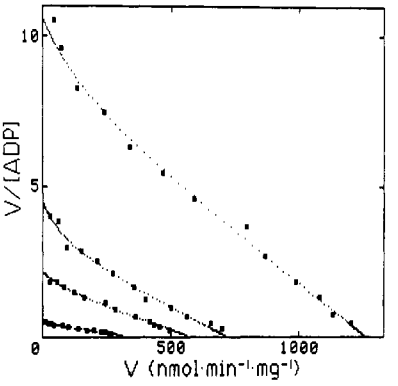

FIGURE 5: Effect of increasing concentrations of FCCP on the kinetics of ATP synthesis as catalyzed by SMP pretreated with DCCD to partially inactivate the ATP synthase complexes. Assay conditions were as described under Materials and Methods, except that SMP concentration was 42 μg/mL. The respiratory substrate was 0.5 mM NADH, and the variable substrate was ADP (4–3900 μM). The ATPase activity of SMP was inhibited by 78% by treating the particles with 15 μM DCCD for 190 min at 0 °C. FCCP was added to the reaction mixtures at a concentration (from top to bottom) of 0, 50, 100, and 200 nM.

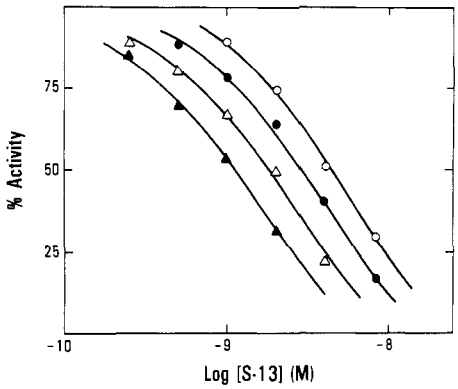

FIGURE 6: Effect of attenuation of respiration rate on the I_{50} of S-13 to uncouple ATP synthesis. Assay conditions for ATP synthesis were as given under Materials and Methods. The respiratory substrate was 0.5 mM NADH. The rate of NADH oxidation was partially inhibited by addition of different concentrations of Seconal to the reaction mixtures. Seconal concentrations (millimolar) and rates of ATP synthesis (nanomoles of ATP formed per minute per milligram of protein) in the absence of S-13 were, respectively, 0 and 2334 (○), 0.2 and 1759 (●), 0.4 and 1036 (△), and 0.7 and 383 (▲). S-13 was added to the reaction mixtures as indicated.

III suggest that, at still higher ratios of the rate of energy production relative to the number of functional F_oF_1 complexes and greater degrees of uncoupling, the apparent K_m^{ADP} may continue to increase. Similar results were obtained with P_i as the variable substrate and NADH as the energy source. Using a DCCD-treated SMP preparation in which ATPase activity had been inhibited by 78%, the apparent high $K_m^{P_i}$ increased from 2.86 mM in the absence of FCCP to 7.22 mM in the presence of 200 nM FCCP (data not shown).

Effect of Partial Inhibition of the Respiratory Chain or the ATP Synthase Complexes on the I_{50} of Uncouplers. The experiments of Figure 5 raised a question that had to be investigated. It is well-known that attenuation of the coupled rate of respiration lowers I_{50} for various uncouplers in oxidative phosphorylation (Kaplay et al., 1970; Terada & Van Dam, 1975). An example of this effect is shown in Figure 6 for S-13. The rationale is that at lower rates of energy input less uncoupler is required to cause the same relative suppression of $\Delta\tilde{\mu}_{H^+}$ and the same degree of uncoupling of the rate of ATP synthesis. Since partial inactivation of the ATP synthase complexes increases the steady-state membrane potential in SMP (Hekman et al., 1988), it was of interest to see what happens to the I_{50} of uncouplers under such conditions. Typical results are shown in Figure 7A (lower panel) with FCCP as the uncoupler and three DCCD-treated SMP samples in which the ATP synthases were inactivated by 52, 74, and 88%, respectively. The upper panel B of Figure 7 shows the same data normalized on the ordinate. It is seen that the effect of partial inactivation of the ATPase complexes is very small on the I_{50} of FCCP. Similar results were obtained with gramicidin D and S-13 as uncouplers and with tributyltin chloride as the ATPase inhibitor (data not shown). We feel that the reason that I_{50} did not increase in these experiments as expected is, at least in part, because in these DCCD-treated preparations the turnover rate for ATP synthesis of the uninhibited F_oF_1 complexes increases, as previously demonstrated (Matsuno-Yagi & Hatefi, 1988). This offsets the effect of partial inactivation of the F_oF_1 complexes insofar as the I_{50} for uncoupling is concerned. For example, with succinate as the respiratory substrate, up to 70% inactivation of the ATPase complexes by either DCCD or tributyltin chloride had essentially no effect on the rate of ATP synthesis per milligram of SMP (Matsuno-Yagi & Hatefi, 1988). In such a system

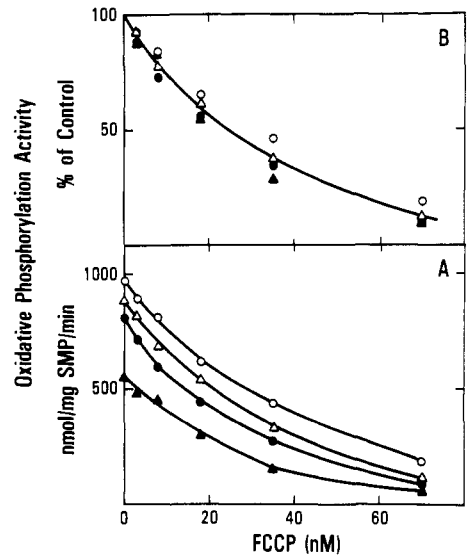

FIGURE 7: Effect of partial inactivation of the ATP synthase complexes on the I_{50} of FCCP for uncoupling ATP synthesis. Assay conditions for ATP synthesis were as described under Materials and Methods. The respiratory substrate was succinate, and SMP concentration was 28 μg/mL. The ATPase activity of SMP was partially inhibited by treating the particles for 190 min at 0 °C with 0 (○), 6.2 (△), 10.4 (●), and 16.7 (▲) μM DCCD. The inhibition of ATPase activity at these DCCD concentrations was 0, 52, 74, and 88%, respectively. FCCP was added to the assay mixtures as indicated. In panel B, the rates of ATP synthesis are expressed on the ordinate as the percent of the rates in the absence of FCCP.

operating at high $\Delta\psi$ in the absence of uncouplers, the balance between the rates of energy input and outflow does not change as increasing fractions of the F_oF_1 complexes are inactivated; hence, the I_{50} for uncouplers remains constant.

Discussion

Effect of Partial Uncoupling and Attenuation of Respiration Rate on the Kinetics of ATP Synthesis. In SMP, both partial uncoupling and attenuation of the rate of respiration diminish the rate of ATP synthesis. Also, both partial uncoupling (regardless of the nature of the uncoupler used) and severe suppression of respiration rate decrease V_{max}/K_m (ordinate intercept of Eadie–Hofstee plots) in oxidative phosphorylation with either ADP or P_i as the variable substrate. Furthermore, in both situations, ln (V_{max}/K_m) decreases linearly with the drop in steady-state $\Delta\psi$ (Hatefi et al., 1982; Hekman et al., 1988). Yet, in spite of these similarities, attenuation of respiration rate and partial uncoupling have very different effects on the apparent K_m values for ADP and P_i in oxidative phosphorylation. As was shown elsewhere (Hekman et al., 1988), attenuation of respiration decreases the apparent K_m^{ADP} to about 2–4 μM;[4] however, partial uncoupling either has no demonstrable effect on the curvilinear kinetics of oxidative phosphorylation (uncoupler: gramicidin D or valinomycin plus nigericin) or shifts the kinetics toward high-K_m values for ADP and P_i (uncoupler: CCCP, FCCP, S-13, or SF6847). Similar results were obtained in ATP-driven reverse electron transfer from succinate to NAD. Partial uncoupling by gramicidin D lowered the rate of reverse electron transfer without changing the apparent K_m for NAD, whereas partial uncoupling by the lipophilic weak acids in-

[4] Because P_i is consumed in the assay of oxidative phosphorylation, the lowest K_m that was technically possible to determine for P_i was ~0.2 mM (Matsuno-Yagi & Hatefi, 1986).

creased K_m^{NAD} as the rate of reverse electron transfer was diminished. We may, therefore, ask what is the reason for the K_m increase and does this K_m change reveal anything about the mechanism of uncoupling?

In the absence of uncouplers, a shift to high K_m for ADP and P_i is observed when the rate of coupled respiration relative to the number of functional ATP synthase complexes is increased. This increase results in two other changes as well: (i) the turnover rate of the ATP synthase complexes in the direction of ATP synthesis increases, and (ii) the P/O ratio at high rates of respiration decreases. With our preparations of SMP oxidizing NADH at 30 °C, the P/O ratio is ~2.0 up to an NADH oxidase rate of about 700 nmol min^{-1} (mg of protein)$^{-1}$ and drops to ~1.5 when the rate of NADH oxidation increases to ≥1200 nmol min^{-1} (mg of protein)$^{-1}$. One possible interpretation of these changes is that increased protonic energy flux through the ATP synthase complex (a) results in high apparent K_m values for ADP and P_i and (b) leads to a higher degree of slippage (Zoratti et al., 1986; Kamp et al., 1988) in the coupling mechanism within the ATP synthase complex. This interpretation provides a ready explanation for the results described above with the lipophilic weak acid uncouplers. Thus, it is conceivable that these reagents react with the F_oF_1 complexes and increase the rate of uncoupled energy flux (slip) through F_oF_1. The ATP synthase "sensing" the high energy flux shifts to higher K_m modes, even though mechanistic slip results in a decreased rate of ATP synthesis. This explanation is fully consistent with the results of Figure 5 in which the apparent K_m^{ADP} increased to 800 μM. In these experiments, SMP had been pretreated with DCCD in order to inactivate a large fraction of the ATP synthase molecules. Studies reported elsewhere had shown that under these conditions (a) energy flux through the remaining, active F_oF_1 units greatly increases and results in very high rates of ATP synthesis per F_oF_1 complex (440 s^{-1}) (Matsuno-Yagi & Hatefi, 1988) and (b) inactivation of the ATP synthase complexes of SMP up to 94% does not increase the apparent K_m^{ADP} beyond ~130 μM (Hekman et al., 1988), suggesting that there might be a normal mechanistic limit to K_m^{ADP} increase as a result of increased turnover rate of the F_oF_1 complexes. Now, such DCCD-treated SMP, containing a limited number of active F_oF_1 complexes under a high protonmotive force and capable of rapid turnover in the direction of ATP synthesis, are subjected in the experiments of Figure 5 to the action of an uncoupler such as FCCP. The uncoupler introduces a slip in the rapidly turning over coupling reaction within the active ATP synthase complexes, thereby greatly increasing the uncoupled rate of energy flux through these units. As a result, the apparent K_m for ADP and P_i increases, and, at any given uncoupler concentration, the greater the rate of respiration relative to the number of active F_oF_1 units, the higher these apparent K_m values.

An important implication of the above interpretation of the results is that uncouplers (especially the lipophilic weak acids) interact with the energy-transducing enzyme complexes of mitochondria and increase slippage in the intracomplex coupling mechanism for product synthesis (e.g., ATP in oxidative phosphorylation or NADH in energy-driven reverse electron transfer). This possibility is consistent with reports regarding the existence of uncoupler binding sites in SMP (Hanstein, 1976; Kurup & Sanadi, 1977; Katre & Wilson, 1977, 1978; Hatefi, 1980; Terada et al., 1988), and regarding uncoupler-resistant bacterial mutants (Decker & Lang, 1977, 1978; Guffanti et al., 1981; Jones et al., 1986). However, it is important to emphasize in conclusion that we do not propose that the above mechanism of uncoupling occurs to the exclusion of, or supersedes in efficiency, the classical chemiosmotic mechanism of uncoupling.

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Inactivation of DNA Polymerase I (Klenow Fragment) by Adenosine 2′,3′-Epoxide 5′-Triphosphate: Evidence for the Formation of a Tight-Binding Inhibitor†

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ABSTRACT: The suicidal inactivation of *Escherichia coli* DNA polymerase I by epoxy-ATP has been previously reported (Abboud et al., 1978). We have examined in detail the mechanism of this inactivation utilizing a synthetic DNA template-primer of defined sequence. Epoxy-ATP inactivates the large fragment of DNA polymerase I (the Klenow fragment) in a time- and concentration-dependent manner (K_I = 21 μM; k_{inact} = 0.021 s^{-1}). Concomitant with inactivation is the incorporation of epoxy-AMP into the primer strand. The elongated DNA duplex directly inhibits the polymerase activity of the enzyme (no time dependence) and is resistant to degradation by the 3′ → 5′ exonuclease and pyrophosphorylase activities of the enzyme. Inactivation of the enzyme results from slow (4×10^{-4} s^{-1}) dissociation of the intact epoxy-terminated template-primer from the enzyme and is thus characterized as a tight-binding inhibition. Surprisingly, while the polymerase activity of the enzyme is completely suppressed by epoxy-ATP, the 3′ → 5′ exonuclease activity remains intact. The data presented demonstrate that even though the polymerase site is occupied with duplex DNA, the enzyme can bind a second DNA duplex and carry out exonucleolytic cleavage.

DNA polymerase I of *Escherichia coli* (Pol I)[1] is a multifunctional enzyme involved in the replication and repair of DNA in vivo (Kornberg, 1980). Replication proceeds through the 5′ → 3′ polymerization of dNTP's onto a primer strand directed by a DNA template. The fidelity of this duplication is ensured by an associated 3′ → 5′ exonuclease activity that removes errors introduced by the polymerase. The enzyme also possesses a distinct 5′ → 3′ exonuclease activity that is involved in nick translation during DNA repair (Setlow & Kornberg, 1972). Limited proteolytic digestion of Pol I (103 kDa) yields a large fragment, the Klenow fragment (68 kDa), which contains polymerase and 3′ → 5′ exonuclease activities but lacks the 5′ → 3′ exonuclease activity (Klenow & Henningsen, 1970; Brutlag et al., 1969).

It has long been appreciated that the polymerase and 3′ → 5′ exonuclease activities constitute discrete catalytic sites. This was first suggested by the selective inhibition of polymerase (Que et al., 1979) and 3′ → 5′ exonuclease (Que et al., 1978)

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