PHOSPHORYLATION COUPLED TO OXIDATION OF THIOL GROUPS (GSH) BY CYTOCHROME C WITH DISULFIDE (GSSG) AS AN ESSENTIAL CATALYST* III. UNCOUPLING AND UNCOUPLER-DEPENDENT ATPASE

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SUMMARY

Phosphorylation coupled to electron transfer from GSH to cytochrome c is uncoupled by 10 μM 2,4-dinitrophenol, 10 μM dicoumarol, 3 μM pentachlorophenol, and 1 μM carbonyl-cyanide-m-chlorophenylhydrazone under anaerobic conditions, and by oxygen. When the uncouplers are added 2 minutes before the enzymatic trapping system, all of the ATP disappears. Added ATP is hydrolyzed within 2 minutes in uncoupler-dependent ATPase reversal of the phosphorylation reaction. These characteristics of the system emphasize the fact that oxidative phosphorylation in mitochondria may depend on the same fundamental properties of thiol and disulfide.

The coupling of ATP formation to the transfer of electrons from GSH to cytochrome c in the presence of GSSG, as demonstrated in the preceding papers (1), was postulated and tested because the unusual properties of the GSSG + GSH mixture (2) made the thiol-disulfide interaction a likely candidate for facilitating electron transfer into and out of cytochromes and simultaneously presented the possibility of energy conservation. Once this was demonstrated, it became important to examine the sensitivity of the reaction to several agents which are true uncouplers in mitochondrial oxidative phosphorylation. Insensitivity to uncoupling

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agents has been the basis for discarding a number of proposed mechanisms. As in the preceding papers, ATP was measured by NADPH formation with an enzymatic trapping system (1).

Actually, there is some disagreement as to how uncouplers might act, and they may not all act by the same mechanism (3). In this communication we report the fact that the phosphorylation coupled to electron transfer from GSH to cytochrome c in the presence of GSSG using purified materials is sensitive to uncoupling agents at the same concentrations as oxidative phosphorylation in mitochondria. In addition, the phosphorylation reaction can be studied in the reverse direction as an uncoupler-dependent ATPase. This means that direct reaction with a chemical intermediate, a hypothesis for which evidence has appeared (4,5), must be considered for the mechanism of true uncouplers, along with increased proton conduction (6) and transport by mitochondrial membrane carriers (7).

Indications that the reaction ran rapidly in the reverse direction came from experiments in which the generated ATP disappeared when O_2 was admitted before the enzymatic trapping system was added. Therefore, most of the ATPase experiments had to be run under strict anaerobiosis. Substances used in the uncoupling and ATPase experiments were 2,4-dinitrophenol (DNP) 10-15 μM, dicoumarol 10 μM (DiC), pentachlorophenol (PCP) 3-5 μM, carbonylcyanide-m-chlorophenylhydrazone (Cl-CCP) 1 μM.

The effect of uncouplers on the yield of NADPH (equivalent to one $\sim$P as ATP with the specific enzymatic trap) is shown in Table I. When uncoupler is present initially with all other reaction components with the exception of GSH, which is added to start the reaction, no generation of ATP occurs. The possibility that uncouplers like Cl-CCP might inhibit the enzymes in the trap-

TABLE I

UNCOUPLING OF THE PHOSPHORYLATION COUPLED TO OXIDATION OF GSH + GSSG BY CYTOCHROME c

ATP was measured by NADPH formation by the trapping system, hexokinase + glucose + glucose-6-phosphate dehydrogenase, and NADP. Cytochrome c was all reduced within 2 min. All cuvettes contained 45 mM Tris·HCl pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 4.5 mM $MgCl_2$, 25 mM glucose. The cuvettes and solutions were flushed thoroughly with nitrogen. The reaction was started by adding GSH which had been placed in the plunger compartment of The American Instrument Co. anaerobic cuvettes. NADPH was measured by A_{340} in a Zeiss spectrophotometer, 1 cm light path. Cytochrome c was placed in the blank cuvette and the balance adjusted so that initial readings on experimental cuvettes were 0-0.1. The absolute A_{340} was about 0.250 higher than the initial reading. 25° C.

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10	11
Cytochrome c 27.4μM	+	+	+	+	+	+	+	+	+	+	+
GSH 1 mM	+	+	+	+	+	+	+	+	+	+	+
GSSG 11 mM	+	+	+	+	+	+	+	+	+	+	+
PO_4 1 mM	-	+	+	+	+	+	+	+	+	+	+
ADP 400 μM	+	+	+	+	+	+	+	+	+	+	+
NADP 0.35 mM	+	+	+	+	+	+	+	+*	+	+	+
Hexokinase 90 μg/ml	+	+	+	+	+	+	+	+	+*	+*	+
G-6-PDH* 0.7 μg/ml	+	+	+	+	+	+	+	+	+	+	+*
DNP 10 μM	-	-	+	-	-	-	-	-	-	-	-
Cl-CCP 1 μM	-	-	-	+	-	-	-	-	-	+	+
PCP 5 μM	-	-	-	-	+	-	-	-	-	-	-
Dicumarol 10 μM	-	-	-	-	-	+	-	-	-	-	-
Oxygen (Air)	-	-	-	-	-	-	-	+*	-	-	-
Ethanol 33 mM	-	-	-	-	-	-	+	-	-	-	-
Initial A_{340} x 1000	026	012	160	070	010	071	025	038	022	008	028
Started with GSH											
A_{340} x 1000 1 min	038	142	180	088	018	060	025	049	155	032	072
" " 2 min	-	152	200	-	020	052	-	048	159	032	-
" " 3 min	040	154	-	086	020	052	026	-	159	032	100
" " 5 min	040	154	200	084	018	052	026	047	159	032	103
ΔA_{340} x 1000	14	140	40	18	10	-19	1	11	137	24	75
ATP found μM	2.1	22	6.4	3	1.5	0	0	1.7	22	3.8	12

* In No. 8 air was admitted 3 min after GSH addition, and the ATP measuring system added 1 min later. In No. 9 the trapping enzymes were added 4 min after GSH. In Nos. 10 and 11 the trapping enzymes and the Cl-CCP were added together 3 min after GSH. This last procedure resulted in partial inactivation of the ATP measuring enzymes while exposed to relatively concentrated Cl-CCP in the cap of the anaerobic cuvette. DNP, 2,4-dinitrophenol; Cl-CCP, carbonyl cyanide m-chlorophenylhydrazone; PCP, pentachlorophenol; G-6-PDH, glucose-6-phosphate dehydrogenase.

ping system during exposure before initiation of the reaction with GSH has been ruled out. No ATP is formed if uncoupler is

present, the reaction is started with GSH, and the ATP measuring trap is added 2-4 min later, after cytochrome c reduction is complete. Energy conservation as ATP was very good with such a sequence without uncoupler (1). When 100-fold greater levels of Cl-CCP were present with the trapping enzymes in the cap of the cuvette during gassing with N_2, and added together, the trap had been partially inactivated. With hexokinase in the cap the competing ATPase reaction predominated. With hexokinase in the cuvette, the altered trap recorded some glucose-6-phosphate.

Uncoupler added after glucose-6-phosphate formation has occurred has no influence on the ΔA_{340} nm (yield of ATP). This adds additional weight to the multiple lines of evidence that the ΔA_{340} is due to NADPH formation and not a ΔA_{340} in any other reaction component, intermediate, or product.

The uncoupler-dependent ATPase was studied in three different ways. In each case anaerobiosis was carefully maintained and the complete GSSG + GSH + cytochrome c + P_i + $MgCl_2$ + EDTA + BSA + Tris buffer system was present and the cytochrome c was reduced. In the first method ATP was generated from ADP to 20 μM levels by the GSH-cytochrome c reaction, and uncoupler was added to one of a pair of cuvettes 2 minutes before the enzymatic trap was added to both cuvettes. The expected amount of NADPH was formed in the control, but none was formed where uncoupler had been added (Table II). The ATP had disappeared. If the uncoupler effect were merely an artifact in A_{340}, or an inhibition of the enzymes, complete inactivation of the trap system would be required to make the ATP non-measurable. Experiments with added ATP indicate that the trap is still active.

In the second procedure 20 μM ATP was generated from ADP by the complete system and 20-50 μM of known ATP was added at the

TABLE II

UNCOUPLER-DEPENDENT ATPASE ACTIVITY OF SYSTEM WHICH COUPLES PHOSPHORYLATION TO OXIDATION OF GSH + GSSG BY CYTOCHROME c

ATP measurements and experimental methods were similar to those in Table I. All cuvettes contained 45 mM Tris·HCl pH 7.6, 1 mg/ml BSA, 1 mM EDTA, 4.5 mM $MgCl_2$, and 11 mM glucose. After anaerobiosis had been carefully established, GSH was added and 3 min allowed for generation of ATP. Then in No. 5-11, larger amounts of exogenous ATP were added. Uncouplers were added where indicated, and 2 min later, or 5 min after GSH, the ATP measuring enzyme system was added (Brackets). No. 11 is a control with added ATP only (No ADP present). Additional ATP after cuvettes are opened to air demonstrates that ATP measuring enzymes are still active in No. 6, where 1 μM Cl-CCP caused disappearance of 45 μM ATP in 2 min anaerobically.

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10	11
Cytochrome c 27.4μM	+	+	+	+	+	+	+	+	+	+	+
GSH 1 mM	-	+	+	+	+	+	+	+	+	+	+
GSSG 11 mM	+	+	+	+	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	+	+	+	+	+	+	+	+
ADP 400 μM	+	+	+	+	+	+	+	+	+	+	-
ATP added μM	-	-	-	-	41	41	41	41	100	100	41
Cl-CCP 1 μM	-	-	-	+	-	+	-	-	-	+	-
DNP 10 μM	-	-	+	-	-	-	+	-	-	-	-
PCP 5 μM	-	-	-	-	-	-	-	+	-	-	-
Hexokinase 90 μg/ml	+	[+	[+	[+	[+	[+	[+	[+	[+	[+	[+
G-6-PDH 0.7 μg/ml	+	+	+	+	+	+	+	+	+	+	+
6-P-GDH* 0.7 U/ml	+	+]	+]	+]	-	-	-	-	-	-	-]
NADP 0.35 mM	+	+	+	+	+	+	+	+	+]	+]	+
A_{340} x 1000 before ATP trap	008	-026	078	032	034	068	160	086	110	078	022
A_{340} x 1000 1 min	008	050	105	028	115	088	200	108	195	100	155
" " 2 min	008	105	105	028	265	105	201	108	410	240	295
" " 3 min	008	225	105	028	355	108	201	108	710	390	297
" " 5 min	008	228	105	028	355	108	201	108	820	480	-
" " 7 min	-	-	-	-	-	-	-	-	-	530	297
ΔA_{340} x 1000	0	254	27	-4	321	40	41	22	710	452	275
ATP found μM*	0	20	4.4	0	51	6.3	6.5	3.5	115	73	44
Aerobiosis + second 41 μM ATP											
ΔA_{340} x 1000					205	244					255
ATP found μM					33	39					41

* 6-P-GDH = 6-phosphogluconate dehydrogenase; when used the trap gives a double yield of NADPH. Actual values for ATP are slightly lower, as there is a small A_{340} 0.01- change due to addition of trapping enzymes.

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end of the electron transfer reaction. All of the ATP disappeared when uncoupler was added 2-3 minutes before the enzymatic trap (Table II).

TABLE III

UNCOUPLER-DEPENDENT ATPASE ACTIVITY OF GSH + GSSG + CYTOCHROME C ANAEROBICALLY. ESSENTIAL COMPONENTS.

ATP measurements and experimental methods were similar to those in Table II, but no ADP was added. All cuvettes contained 45 mM Tris·HCl pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 4.3 mM $MgCl_2$, and 23 mM glucose. After anaerobiosis was established, GSH was added. ATP was added 3 min after GSH. Uncoupler was added immediately after the ATP and 2 minutes before the ATP measuring system (Brackets).

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8*	9	10	11
Cytochrome c 24.4μM	-	+	+	+	+	+	+	+	-	+	+
GSSG 11 mM	-	+	+	+	+	+	+	+	+	-	+
GSH 1 mM	-	+	+	+	+	+	+	+	+	+	-
PO_4 1 mM	+	+	+	+	+	+	+	+	+	+	+
ATP 50 μM	+	+	+	+	+	+	+	+	+	+	+
DNP 10 μM	-	-	+	-	-	-	+	-	-	-	-
Cl-CCP 1 μM	-	-	-	+	-	+	-	+	+	+	+
Oxygen (Air)	-	-	-	-	+	-	-	+	-	-	-
Hexokinase 90μg/ml	[+	+	+	+	+	+	+	+	+	+	+
G-6-PDH 0.7 μg/ml	+	+	+	+	+	+	+	+	+	+	+
NADP 0.35 mM	+]	+]	+]	+]	+]	+]	+]	+]	+]	+]	+]
A_{340} x 1000 before ATP trap	043	032	120	090	025	068	110	046	038	048	012
A_{340} x 1000 0.5 min	105	175	121	120	025	088	150	305	305	335	320
" " 2 min	242	300	126	135	026	105	150	355	335	360	325
" " 4 min	295	372	130	137	026	108	150	355	345	360	325
" " 8 min	320	445	133	137	028	108	150	-	345	360	320
" " 10 min	351	445	133	137	028	108	-	-			
$\triangle A_{340}$ x 1000	308	413	13	47	3	40	40	309	307	312	308
ATP found μM*	49	66	2	7.3	0	6.3	6.3	49	49	50	49

* These values are uncorrected for a small A_{340} change usually seen upon addition of the ATP trap, but varying slightly with the composition of the medium. When No. 6 was opened to air and 40 μM ATP added, the A_{340} increased by 0.244 (Equivalent to 39 μM ATP), demonstrating that the ATP measuring enzymes were active where 1 μM Cl-CCP caused disappearance of 43 μM ATP in 1-2 min anaerobically. Although no ADP was added, comparison of No. 2 with No. 1 indicates that electron transfer generated 17 μM ~P which was transferred to ADP as soon as ADP was formed from ATP by the enzymatic trap. No. 8 was aerobic throughout, and the key intermediates were never formed, in contrast to No. 5, where air was admitted after the formation of intermediates and addition of ATP.

In the third procedure the complete system was present except for ADP, so cytochrome c was reduced but there was no generated ATP. The uncoupler-dependent ATPase test was conducted with

added ATP alone. With uncoupler present 25-50 μM ATP all disappeared within 3-4 minutes (Table III). When multiple additions of 25 μM ATP were made, or 75-100 μM in one addition, the ATP did not all disappear, even after 5-7 minutes. The rate of the reaction may not be sufficient to split all of the ATP within this time period. However, other factors may enter the picture. There were indications in some experiments that an uncoupler like Cl-CCP might become inactivated over the longer time period. This may be merely a matter of slow reaction with GSH. In the complete mixture an uncoupler like Cl-CCP is most likely to be reduced or to form a covalent bond with GSH. Another possibility is that higher levels of ATP inhibit ATP removal.

For the phosphorylation coupled to transfer of electrons from GSH to cytochrome c, oxygen also acts as an uncoupler and will initiate ATPase activity. Strict anaerobiosis is essential to study uncoupling as well as phosphorylation. If this type of reaction mechanism functions for the mitochondrial membrane system, it must be in some way protected from O_2.

In general terms, it is interesting that uncoupling in mitochondria might occur (a) by permitting water to reach and hydrolyze an intermediate normally protected in an hydrophobic area, (b) by permitting O_2 to react with an intermediate normally protected from its action, and (c) by direct reaction of uncoupling agents with radical type intermediates. These represent 3 of the direct mechanism possibilities. Numerous indirect possibilities exist. Uncoupling agents may penetrate the barrier which normally protects radical forms from O_2 or OH^-. That many of the classical uncoupling agents are compounds which could interrupt reactions with radical intermediates has been pointed out a number of times over the years (8-12). Unstable radical inter-

mediates are also proposed in the hypothesis of Wang (13). Other workers have discussed the possible role of Pi electron interaction and charge transfer complexes (14,15).

Additional evidence for a radical intermediate comes from the fact that small amounts of ethanol, for example 5 µl in 3 ml of aqueous medium, completely uncouples the phosphorylation. While this might be considered a small amount of alcohol, actually the final concentration is 33 mM. In the GSH-cytochrome c model system, great caution must be exercised in drawing conclusions concerning effects of substances which must be dissolved in alcohol to make a stock solution.

Several points are worthy of special note. Not only do the uncouplers act in catalytic amounts, for example 1 µM Cl-CCP or 5 µM PCP in a system which contains 1 mM P_i and 0.4 mM ADP and would normally generate 20 µM ATP, but their rapid reaction with the intermediate responsible for phosphorylation is emphasized by the fact that they uncouple in the presence of 1 mM GSH. At least during the 2 to 4 minutes needed for the oxidative phosphorylation the uncoupler does not become inactivated.

The phosphorylation in this system does not show the equivalent of respiratory control seen in mitochondria. The rate of electron transfer from GSH to cytochrome c, under the conditions studied so far, is not altered by the presence or absence of phosphate, AMP, or ADP, or uncoupler. Respiratory control is usually much lower in submitochondrial particles. Therefore, the lack of respiratory control in this simplified model system, even if it is the prototype for oxidative phosphorylation mechanisms, is not surprising. In mitochondria other reactants and membrane structure undoubtedly could impose additional characteristics on the basic system. Discussion of possible reaction mechanisms will be considered in the following paper (V).

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