PHOSPHORYLATION COUPLED TO OXIDATION OF THIOL GROUPS (GSH) BY CYTOCHROME C WITH DISULFIDE (GSSG) AS AN ESSENTIAL CATALYST*

II. DEMONSTRATION OF ATP FORMATION FROM ADP AND HPO_4^{2-}

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SUMMARY

The oxidation of GSH by cytochrome c in buffered solution with GSSG as a catalyst can generate ATP from ADP and inorganic phosphate. The ATP formation is not due to adenylatekinase activity in the ATP measuring enzyme system or other components. This direct coupling of phosphorylation with electron transfer is selective for ADP, with 0.15 mM being comparable to 10 mM AMP. It may represent the type reaction for coupling of phosphorylation to one or more sites in the electron transport chain in mitochondria. The stoichiometry indicates one $\sim PO_4$ per cytochrome c reduced or electron transferred. Metal catalyzed transfer of electrons from GSH to cytochrome c does not produce phosphorylation.

Studies on the coupling of phosphorylation to electron transfer from GSH to cytochrome c have been extended to ADP, which is probably the primary acceptor within mitochondria, although these organelles have adenylatekinase between the outer and inner membrane. The system for conversion of ADP + P_i to ATP is illustrated in Table I. It is the same as that used in the preceding paper (1) except for the phosphate acceptor and omission of adenylatekinase. The essential nature of various components is established by single omission experiments. The

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requirements are GSH, GSSG, cytochrome c, P_i, ADP, hexokinase, Mg^{++}, glucose, glucose-6-phosphate dehydrogenase and NADP. Only GSSG + GSH are essential to reduce cytochrome c rapidly. Experimental details are given in the legends to the Tables.

To establish the validity of this conversion of ADP + P_i to ATP it was absolutely essential to demonstrate that there was no adenylatekinase activity in the system, either as an impurity in the trapping enzymes used or inherent in the intermediate mechanisms of coupling of phosphorylation to GSSG catalyzed transfer of electrons from GSH to cytochrome c. Preliminary indications of this were found in the preceding study with AMP (1). However, in those experiments the ADP concentration could never have exceeded 25 μM, and in the complete system with trapping enzymes it would have been much lower. It was essential to demonstrate the absence of adenylatekinase activity with the 350 μM levels of ADP present in routine experiments when added ADP was used as acceptor.

In the single omission experiments presented in Table I, the systems which were complete except for P_i, GSH, GSSG, or cytochrome c did not show ATP formation from added ADP. The experiments without GSSG were important to demonstrate that there was not an adenylatekinase activity which was inactivated by GSSG, but reactivated upon the addition of GSH, the procedure for initiating most of the experiments. No partial system showed any measurable adenylatekinase activity. ATP was formed only with the complete system, and if this were due to an adenylatekinase type of reaction, it would strangely require P_i, be stoichiometrically determined by the amount of cytochrome c, and be abolished by uncouplers (See Paper III of this series (2). One can speculate about cytochrome c being required to generate GS^+,

TABLE I

FORMATION OF ATP FROM ADP + P_I DURING OXIDATION OF GSH + GSSG BY CYTOCHROME C. ESSENTIAL COMPONENTS.

ATP generated was measured by NADPH formation by the trapping system, hexokinase + glucose + glucose-6-phosphate dehydrogenase, and NADP. Cytochrome c was all reduced within 2 minutes. In addition to the components listed all cuvettes contained 45 mM Tris. HCl pH 7.6, 1 mM EDTA, 1 mg/ml BSA. 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. Temp. 25°.

Components and final concentration	Cuvette No.										
	1	2	3	4	5	6	7	8	9	10	11
Cytochrome c 24 µM	+	+	-	+	+	+	+	+	+	+	+
GSH 1 mM	-	+	+	+	+	+	+	+	+	+	+
GSSG 10 mM	+	+	+	-	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	+	-	+	+	+	+	+	+
ADP 0.4 mM	+	+	+	+	+	-	+	+	+	+	+
Hexokinase* 90µg/ml	+	+	+	+	+	+	-	+	+	+	+
$MgCl_2$ 4.5 mM	+	+	+	+	+	+	+	-	+	+	+
Glucose 25 mM	+	+	+	+	+	+	+	+	-	+	+
G-6-PDH* 0.5µg/ml	+	+	+	+	+	+	+	+	+	-	+
NADP 0.35 mM	+	+	+	+	+	+	+	+	+	+	-
A_{340} x 1000 before GSH	038	012	034	098	098	090	066	013	028	080	000
Started with GSH											
A_{340} x 1000 at 1 min	042	152	041	110	103	084	066	002	006	086	-016
" " 2 min	042	152	041	110	103	084	066	002	014	086	-018
" " 5 min	042	154	041	110	103	084	066	000	014	086	-018
ΔA_{340} x 1000	4	140	7	12	7	-6	0	-13	-22	6	-18
Calculated µM ~P	0	23	0	0	0	0	0	0	0	0	0

* The sources of enzymes were as follows: Cytochrome c, Sigma Type III and VI; hexokinase, Sigma V and Boehringer; glucose-6-phosphate dehydrogenase (G-6-PDH), Sigma and Boehringer; glutathione reductase, Sigma and Boehringer. The sources of other materials were: GSH, Calbiochem and Sigma; GSSG, ADP, ATP, and NADP, Sigma; $MgCl_2$, glucose, K_2HPO_4, and EDTA, Mallinckrodt A.R.; Tris, Sigma A.R.; BSA, Armour crystalline bovine serum albumin.

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which might show adenylatekinase activity, and even about uncouplers reacting with essential intermediates, but a requirement for P_i is most difficult to visualize. The new information must be added to the fact that added adenylatekinase is an absolute re-

quirement when AMP is used as acceptor. The evidence seems very much against an adenylatekinase activity being responsible for the ATP formation.

One could ask why added adenylatekinase was necessary at all in the preceding study on AMP + P_i if ADP + P_i will go to ATP, as demonstrated in this paper. The reason lies in the low levels of ADP reached (20 μM) with AMP + P_i. In Table II it can be seen that the measurable ATP formed fell off rapidly as the added ADP concentration was reduced below 100 μM.

Variations in the concentrations of several components are shown in Table II. The requirements are the same as for the AMP + P_i system, except, of course, that no adenylatekinase enzyme is needed. Remarkable is the fact that the system is virtually optimal with 100 μM ADP. The product of the trapping system, NADPH,

TABLE II

FORMATION OF ATP FROM ADP + P_I DURING OXIDATION OF GSH + GSSG BY CYTOCHROME C. OPTIMAL AMOUNTS OF ADP, GSH AND GSSG

ATP measurement and the experimental procedure were as described in Table I. In addition to the components listed each cuvette contained 45 mM Tris·HCl pH 7.6, 1 mM EDTA, 1 mg/ml BSA, 4.5 mM $MgCl_2$, 25 mM glucose, hexokinase 90 μg/ml, glucose-6-P dehydrogenase 0.7 μg/ml, and NADP 0.35 mM. The concentration is entered in the table where a component was varied.

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10	11
Cytochrome c 27 μM	+	+	+	+	+	+	+	+	+	+	+
GSH 1 mM	-	+	+	+	+	+	+	+	0.2	0.5	2.0
GSSG 10 mM	+	+	+	+	+	+	2.0	5.0	2.0	+	+
PO_4 1 mM	+	+	+	+	+	+	+	+	+	+	+
ADP 0.4 mM	+	+	0.1	.05	1.0	2.0	+	+	+	+	+
Initial A_{340} x 1000	020	028	030	038	056	046	038	041	038	038	038
Started with GSH											
A_{340} x 1000 1 min	028	163	159	099	105	084	064	088	046	070	105
" " 2 min	029	175	165	100	125	090	098	104	038	074	115
" " 3 min	029	175	169	100	145	090	098	128	038	076	120
" " 5 min	029	175	169	100	148	090	098	130	038	078	120
$\triangle A_{340}$ x 1000	9	147	139	62	92	44	60	91	0	40	82
Calculated μM ~P	0	24	23	10	15	7	9.7	15	0	6.4	13

TABLE III

FORMATION OF ATP FROM ADP AND P_I DURING OXIDATION OF GSH + GSSG BY CYTOCHROME C. ESSENTIAL COMPONENTS AND 2 STEP REACTION EXPERIMENTS.

Because of the unusual nature of the reaction additional controls are presented to show the absence of ATP formation whenever components are omitted, and the reaction is separated into 2 stages (a) generation during cytochrome c reduction, and (b) trapping ATP for measurement after cytochrome c reduction. Except as indicated the experiments were conducted as in Table I.

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10	11
Cytochrome c 27 μM	+	+	+	+	+	+	+	+	+	+	+
GSH 1 mM	-	+	+	+	+	+	+	+	+	+	+
GSSG 10 mM	+	+	+	+	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	+	+	+	+	+	+	+	+
ADP 0.4 mM	+	+	+	+	+	+	+	+	+	+	+
$MgCl_2$ 4.5 mM	+	+	+	+	+	+	+	+	+	+	+
Glucose 25 mM	+	+	+	+	+	+	[+*	[+*	+	+	+
Hexokinase 90μg/ml	+	+	+	+	+	+	+	+	[+*	[+*	+
G-6-PDH* 0.7μg/ml	+	+	+	+	+	-	+	+]	+	+	[+*
6-P-GDH* 0.4U/ml	+	+	-	+	+	+	+]	-	+	+]	+
NADP 350 μM	+	+	+	+	+	+	+	+	+]	+	+]
Tris pH 7.6 45 mM	+	+	-*	+	+	+	+	+	+	+	+
EDTA 1 mM	+	+	+	-	+	+	+	+	+	+	+
BSA 1 mg/ml	+	+	+	+	-	+	+	+	+	+	+
A_{340} x 1000 before GSH											
Started with GSH	068	044	017	036	066	080	058	032	020	058	030
A_{340} x 1000 1 min	088	240	120	270	280	088	140	120	100	205	240
" " 2 min	091	305	125	290	300	086	295	162	200	255	250
" " 4 min	093	350	137	290	300	086	335	163	258	295	258
" " 6 min	093	350	137	290	300	086	335	163	258	307	258
$\triangle A_{340}$ x 1000	25	306	120	254	234	8	277	131	238	249	228
Calculated μM ~P	2	24	19	20	19	0	22	21	19	20	18
GSSG reductase A_{340} at 2 min		210		170	100						
5 min		130		048	080						
10 min		040		048	080						

* The bracketed components were added 3-4 min after GSH, 1-2 min after the cytochrome c was all reduced. G-6-PDH = glucose-6-phosphate dehydrogenase. 6-P-GDH = 6 phosphogluconate dehydrogenase. When Tris was omitted phosphate was the only buffer.

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has again been identified enzymatically with glutathione reductase (Table III).

With ADP, P_i, GSH, GSSG, and cytochrome c being absolute re-

quirements, with NADPH being identifiable enzymatically with glutathione reductase, with NADPH (ΔA_{340}) being formed only when trapping enzymes are present, and with the specificity of hexokinase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, it is difficult to draw any conclusion other than that direct phosphorylation of ADP to ATP is coupled to electron transfer from GSH to cytochrome c. Added known ATP demonstrates that the ATP measuring system is functioning as predicted, and the specificity and equivalence of NADPH formation in relation to ATP is clear cut with added ATP.

Additional evidence that NADP, glucose, and the trapping enzymes are not involved in the primary steps in the energy conserving reaction is available. In pure solution NADP is not reduced by a GSSG + GSH mixture with or without cytochrome c. The reaction can be carried out in 2 steps: (a) reduction of cytochrome c and generation of ATP, and (b) late addition of the NADP, glucose and trapping enzymes several minutes after reduction of cytochrome c is complete. As the experiments presented in Table III indicate, the ATP generated is essentially the same as when the trap is present during electron transfer (See also paper IV (3)). This indicates that ATP is formed in substantial amounts and its detection is not based on trapping each molecule as it is formed, i.e., from an equilibrium with a very unstable intermediate, an equilibrium far in the opposite direction from ATP.

The evidence indicates that synthesis of ATP from ADP + P_i can be coupled to transfer of electrons from GSH to cytochrome c. The selectivity for ADP as compared to AMP is remarkable because of the parallelism with the complete mitochondrial system. The difference between the 0.1 mM ADP and the 5 mM AMP required for near optimal yields is so large that the question of ADP impurity

TABLE IV

STOICHIOMETRIC RELATIONSHIP OF ATP FORMATION TO THE AMOUNT OF CYTOCHROME c USED FOR OXIDATION OF GSH + GSSG

ATP generated was measured by NADPH formation as described in Table I. Cytochrome c was all reduced within 2 minutes. In addition to the components listed, 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. Cytochrome c was placed in the blank cuvette and the balance adjusted so that initial readings on the experimental cuvettes were 0-0.1 for the lower concentrations. Frequently, but not always, cuvettes with higher cytochrome c were read against the same blank. The absolute A_{340} was about 0.250 higher than the initial reading for the routine concentration (20-25 μM) of cytochrome c used.

Component and final concentration	Cuvette No.										
	1	2	3	4	5	6	7	8	9	10	11
Cytochrome c μM	10	49	-	10	18	25	34	48	22	55	75
GSH 1 mM	-	-	+	+	+	+	+	+	+	+	+
GSSG 11 mM	-	+	+	+	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	+	+	+	+	+	+	+	+
AMP 10 mM	+	+	+	+	+	+	+	+	-	-	-
ADP 0.35 mM	-	-	-	-	-	-	-	-	+	+	+
Hexokinase 90μg/ml	+	+	+	+	+	+	+	+	+	+	+
G-6-PDH* 0.7 μg/ml	+	+	+	+	+	+	+	+	+	+	+
Adenylkinase 0.9μg/ml	+	+	+	+	+	+	+	+	-	-	-
NADP 0.35 mM	+	+	+	+	+	+	+	+	+	+	+
A_{340} x 1000 before GSH	038	195	092	072	078	068	300	650	038	002	635
Started with GSH											
A_{340} x 1000 1 min	032	195	-	125	185	130	380	800	145	195	980
" " 2 min	033	195	105	134	185	168	480	890	157	298	1000
" " 5 min	032	195	105	134	185	205	485	915	157	298	1050
ΔA_{340} x 1000	-6	0	13	62	107	137	185	265	119	296	415
Calculated μM ~P	0	0	0	9.9	17	22	29	42	19	47	67

* G-6-PDH = glucose-6-phosphate dehydrogenase.

in the AMP must be raised. Enzymatic tests do not indicate ADP in the AMP, and when AMP is used adenylatekinase is an essential requirement to get ATP. The difference between ADP and AMP may be a matter of reaction rates. The question whether such a difference in rates might be due to orientation of the reacting ADP by the GSSG + GSH complex or by the cytochrome c is a fundamental one for the catalysis and reaction mechanism.

Another remarkable feature of the system is the fact that with 0.1 mM ADP and 1 mM P_i, or 10 mM AMP and 1 mM P_i, so little $\sim$ P is lost in side reactions like PP_i formation. In other model reactions which have been studied PP_i formation has always been appreciable (4-10).

The amount of cytochrome c used determines, within the limits of experimental variation, the amount of ATP which will be formed (Table IV). (The largest other experimental variation is probably in the presence or absence of traces of oxygen after routine gassing with high grade nitrogen.) The relationship is stoichiometric over the range 10 to 75 μM cytochrome c. It retains this relationship with both the AMP and the ADP system. Similar results are obtained at pH 8.0 and at pH 7.6. These findings indicate that the coupling of phosphorylation to oxidation of GSH by cytochrome c is occurring in a specific way, with little loss in side reactions.

The ratio of ATP formed per cytochrome c present and reduced ranges from 0.7 to 1.0, with most of the values being 0.8-0.9. These are uncorrected for the small decrease in A_{340} of some controls. Correction would raise the value slightly. Allowing for a slight loss in side reactions, this stoichiometry means that the system is capable of generating one $\sim PO_4$ per electron transferred to cytochrome c. Although this is astonishing because we had visualized only the possibility of 1 $\sim$P:2e via formation of GS^+, it does not seem to be thermodynamically impossible, since the GSH-GSSG standard oxidation-reduction potential is in the neighborhood of -250 mV and cytochrome c is +250 mV (11). An alternative is that the ratio is 1:2, but that we do not recognize or measure the acceptor for the other electron.

Metal ion catalyzed electron transfer from GSH to cyto-

chrome c, in contrast to that with GSSG, does not result in energy conservation by phosphorylation.

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