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

I. DEMONSTRATION OF ADP FORMATION FROM AMP AND HPO_4^{2-}

Audrey A. Painter and F. Edmund Hunter, Jr.

From

The Edward Mallinckrodt Department of Pharmacology,
Washington University School of Medicine,
Saint Louis, Missouri 63110 U.S.A.

Received June 10, 1970

SUMMARY

The oxidation of GSH by cytochrome c in buffered solution in the presence of EDTA requires GSSG as a catalyst. It appears that an intermediate complex between GSSG and GSH is more active in reducing cytochrome c. During this electron transfer reaction ADP can be generated from AMP and inorganic phosphate. AMP and inorganic pyrophosphate yield ATP. This reaction, which uses common biological materials and occurs in aqueous medium, is a most interesting model for oxidative phosphorylation. It may involve the basic mechanism for coupling of phosphorylation to electron transport in mitochondria.

Some years ago mixtures of GSSG and GSH were studied extensively with respect to their ability to induce lipid peroxidation and swelling-disintegration in mitochondria (1, 2) and with pure lipids (3). The mixture of GSSG and GSH seemed to have special properties. In further work it was observed that relatively low concentrations of phosphate and arsenate prevented the lipid peroxide producing effect of GSSG + GSH both with mitochondria (4) and with pure lipids (3). The possible reaction of P_i and arsenate with an intermediate reactive complex of GSSG + GSH was considered and the possibility of a biological role was pointed out

* This work was supported by the Department of Health, Education, and Welfare, Grant No. AM-11673-03 from the National Institute for Arthritis and Metabolic Diseases, and Grant No. HE-10107-04A from the National Heart Institute, U. S. Public Health Service.

(4). However, since EDTA also interrupted lipid peroxidation induced by GSSG + GSH, it was not possible to be certain that the P_i and arsenate were doing anything beyond complexing traces of Fe^{2+} needed to form and/or decompose lipid peroxides. Later work indicated that both cytochrome c (5) and non-heme Fe (unpublished work) were involved in the complete sequence of formation and breakdown of lipid peroxide in mitochondria.

Studies of the reduction of cytochrome c in pure solution by GSSG + GSH mixtures led to the demonstration that there are two reactions for reduction of cytochrome c by GSH (6-9). One is metal catalyzed and greatly inhibited by physiological ionic strength. The other is GSSG catalyzed, is completely insensitive to changes in ionic strength, and is faster anaerobically than aerobically. The reaction is not limited to glutathione, as certain other disulfides and thiols show similar behavior (9). The reaction is catalyzed in direct proportion to GSSG. It is not due to metal ion or a metal-GSSG complex. Our working hypothesis for the mechanism of this reaction (9) includes interaction between GSSG and the initial GS^- as well as the GS· and GS^+ in a way which facilitates electron transfer and stabilizes both the first and the second oxidation products, possibly by electron sharing in a ring of three sulfur atoms. The product GS^+ might be involved in energy conservation reactions. The stabilization might be essential to give time for the GS^+ to interact with phosphate before it is lost by interaction with H_2O, OH^-, GS^-, or GS·.

GSSG probably catalyzes reaction of GSH with oxygen also, as there is a considerable reaction that is not eliminated by EDTA (10), and the reduction of cytochrome c is more rapid if competing oxygen is removed (9). Moreover, reduction of cytochrome c,

cytochrome a, and cytochrome b in intact mitochondria is extremely slow with GSH, rapid with GSSG + GSH (11). EDTA was used in the latter experiments to prevent lipid peroxidation in the mitochondria. In the absence of EDTA (which prevents P_i-induced swelling), with lipid peroxidation suppressed by 5 mM P_i, GSSG + GSH appears to serve as a substrate for energy dependent P_i-induced swelling of mitochondria (12).

We have now studied the GSSG catalyzed oxidation of GSH by cytochrome c in the presence of P_i and AMP, and PP_i and AMP, along with an enzymatic system for trapping ADP or ATP. ATP synthesis is followed by NADPH formation (increase in A_{340} nm) in a system consisting of hexokinase, adenylatekinase if P_i is used, glucose, glucose-6-phosphate dehydrogenase and NADP. In some experiments 6-phosphogluconate dehydrogenase was also added to double the amount of NADPH derived from each glucose-6-phosphate formed. Whether a single or double yield of NADPH is desired is largely a matter of convenience, but it does minimize errors in A_{340} readings and add specificity to identification of the products. Most experiments were conducted with a Zeiss spectrophotometer, with cytochrome c in the blank cuvette against which the initial 340 nm reading was balanced. Some experiments were done with an American Instrument-Chance dual wavelength spectrophotometer 340-371 nm. The details of the methods are presented in the legends to the Tables.

The reduction of cytochrome c by GSH + GSSG is rapid under the anaerobic, pH 8.0 conditions used, and with generous supplies of trapping enzymes the phosphorylation keeps pace and stops when the cytochrome c is all reduced, in 1-2 minutes under the conditions used. The only components essential for cytochrome c reduction are GSH and GSSG. The reduction of cytochrome c was not

measured in the routine experiments following A_{340} because of lack of instrumentation for the simultaneous measurements. The amount of the trapping enzymes added routinely was such that the reduction of NADP was also complete in 2-3 minutes. Lower amounts resulted in the ΔA_{340} proceeding at a slower rate, presumably measuring ADP or ATP formed in a more rapid reaction related to cytochrome c reduction by GSH.

TABLE I

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

ADP generated was measured by NADPH formation by the trapping system, adenylatekinase + hexokinase + glucose + glucose-6-phosphate dehydrogenase, and NADP. Cytochrome c was all reduced within 2 min. All cuvettes contained 45 mM Tris·HCl pH 8.0, 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} nm 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°.

Components and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10
Cytochrome c 24 µM	+	+	+	+	+	+	-	+	+	+
GSH 1 mM	-	+	+	+	+	+	+	+	+	+
GSSG 11 mM	+	+	-	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	-	+	+	+	+	+	+
AMP 10 mM	+	+	+	+	-	+	+	+	+	+
Hexokinase 90 µg/ml	+	+	+	+	+	+	+	+	+	-
Adenylatekinase 5µg/ml	+	+	+	+	+	-	+	+	+	+
Glucose-6-PDH 0.5µg/ml	+	+	+	+	+	+	+	+	-	+
NADP 0.3 mM	+	+	+	+	+	+	+	-	+	+
Initial A_{340} x 1000	078	058	038	058	048	048	042	018	056	058
Started with GSH										
A_{340} x 1000 1 min	080	170	032	056	020	020	042	018	056	056
" " 2 min	082	175	034	-	-	020	042	020	-	056
" " 5 min	082	175	034	058	020	020	042	020	052	056
ΔA_{340} x 1000	4	117	-4	0	-28	-28	0	2	0	-2
Calculated µM ~P	0	18.7	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.

In Tables I and II phosphorylation by the complete system at more or less optimal conditions is shown (cuvette 2). The effect of omitting components is compared with the complete system. Table III shows variations in GSH and GSSG concentration.

The evidence indicates ADP formation from AMP + P_i (13) (Table I). Adenylatekinase must be added to get ATP for trapping by the hexokinase reaction. The essential components are cyto-

TABLE II

FORMATION OF ADP FROM AMP + P_I AND ATP FROM AMP + PP_I DURING OXIDATION OF GSH + GSSG BY CYTOCHROME C. ESSENTIAL COMPONENTS AND ENZYMATIC IDENTIFICATION OF THE glucose-6-phosphate AND NADPH FORMED BY ATP TRAPPING ENZYMES

ATP and ADP were measured enzymatically as indicated in Table I. All cuvettes contained 45 mM Tris·HCl pH 8.0, 1 mg/ml BSA, 1 mM EDTA, 24 µM cytochrome c, 10 mM GSSG, 4.5 mM $MgCl_2$, hexokinase 90 µg/ml, 25 mM glucose, glucose-6-phosphate dehydrogenase 0.04 U/ml, and 0.3 mM NADP. Cuvettes and solutions were flushed thoroughly with nitrogen. Temp. 25°.

Component and final concentration	Cuvette No. 1	2	3	4	5	6	7	8	9	10	11
GSH 1 mM	-	+	+	+	+	+	+	+	+	+	+
PO_4 1 mM	+	+	+	+	+	-	-	-	-	-	-
6-P-GDH* 0.4 µg/ml	-	-	-	-	+	-	-	-	-	+	+
AMP 10 mM	+	+	-	+	+	-	+	-	+	+	+
Adenylkinase* 5µg/ml	+	+	+	-	+	-	-	+	+	-	+
Pyrophosphate 1 mM	-	-	-	-	-	+	+	+	+	+	+
Initial A_{340} x 1000	048	048	048	038	088	038	058	050	041	038	042
Started with GSH											
A_{340} x 1000 1 min	048	098	020	040	252	016	100	050	135	094	092
" " 3 min	048	145	020	040	270	016	140	052	170	128	115
" " 5 min	048	160	020	040	285	016	146	053	195	142	194
" " 7 min	048	160	020	040	285	016	150	053	215	181	225
" " 10 min						016	150	053	219	183	267
" " 17 min									219	183	291
ΔA_{340}	0	112	-28	2	197	-22	92	3	178	145	249
Calculated µM ~P	0	18	0	0	16	0	15	0	29	12	20
GSSG Reductase*											
A_{340} x 1000 3 min							130			120	105
" " 5 min							078			070	060
" " 10 min							060			058	-

* The sources of enzymes and other substances were the same as in Table I, with the following addition: 6-phosphogluconate dehydrogenase (6-P-GDH), GSSG reductase (0.6 µg/ml), Sigma.

TABLE III

FORMATION OF ADP FROM AMP + P_I DURING OXIDATION OF GSH + GSSG BY CYTOCHROME C. EFFECT OF O_2, pH, GSH AND GSSG CONCENTRATIONS

In addition to the components listed all cuvettes contained 45 mM Tris-HCl pH 7.6 or pH 8.0, 1 mM EDTA, 1 mg/ml. BSA, 4.5 mM $MgCl_2$, 23 mM glucose, 24 µM cytochrome c, 1 mM P_i, and the ADP trapping enzyme system consisting of hexokinase 90 µg/ml, glucose-6-P dehydrogenase 0.7 µg/ml, adenylate kinase 0.9 µg/ml, and NADP 350 µM. Except for cuvette No. 3 the cuvettes and solutions were flushed thoroughly with nitrogen. The experiments were started by adding the GSH, which was in the plunger cap of the anaerobic cuvette. Temp. 25°.

	pH 7.6		pH 8.0								
			Cuvette No.								
Component and final concentration	1	2	3	4	5	6	7	8	9	10	11
O_2 (Air)	-	-	+	-	-	-	-	-	-	-	-
GSH 1 mM	-	+	+	-	+	0.5	2	+	+	+	+
GSSG 11 mM	+	+	+	+	+	+	+	5	1	0.5	+
AMP 10 mM	+	+	+	+	+	+	+	+	+	+	IMP
Initial A_{340} x 1000	048	042	054	078	076	080	060	080	105	094	048
Started with GSH											
A_{340} x 1000 1 min	048	100	052	082	175	120	160	145	125	110	044
" 3 min	048	164	052	082	188	120	160	145	125	110	035
" " 5 min	048	164	052	082	199	110	158	134	125	110	035
ΔA_{340} x 1000	0	122	-2	4	123	40	100	65	20	16	-13
Calculated µM ~P	0	20	0	0	20	6.4	16	10	3	2.5	0

chrome c, GSH, GSSG, AMP, P_i and the enzymatic trap. The necessity for adding adenylatekinase to get ATP from AMP + P_i indicates that the system does not contain adenylatekinase activity as an impurity in the other enzymes or inherent in the intermediate in the GSH + GSSG + cytochrome c reaction. The absence of NADPH formation with controls containing AMP and enzyme trap, but deficient in some other essential component, indicates that the NADPH formation is not due to small amounts of ADP or ATP in the AMP. AMP was first used at 10 mM with the hope of trapping some energized phosphate. Later experiments indicated that 10 mM is probably optimal but 5 mM sometimes gave equal yields. Reducing the AMP below 3 mM rapidly decreased the yield of ATP.

When PP_i was substituted for P_i, ATP was formed directly from AMP without adenylatekinase being present. The reaction was slower and the yield of ATP was somewhat lower. With adenylatekinase present 2 NADPH are formed per mole of ATP, as would be expected. IMP did not substitute for AMP with either P_i or PP_i.

Since A_{340} was being used as a measure of ATP formed, it was important to establish that the ΔA_{340} was really due to NADPH formation by the trapping enzyme system and not due to some increase in A_{340} because of changes in cytochrome c or formation of some intermediate or product in the reaction. For example, transfer of one electron from GSH to cytochrome c would result in a GS• radical, and some free radicals absorb at 340 nm. Only GSH and GSSG are essential for reduction of cytochrome c, and the A_{340} decreases slightly. However, the presence of P_i and AMP introduced new possibilities for intermediates.

Several lines of evidence indicate that the ΔA_{340} is due to NADPH and not other intermediates. First, no ΔA_{340} is seen if hexokinase, glucose-6-phosphate dehydrogenase or NADP is omitted, with or without P_i and AMP present, but the GSSG + GSH continues to rapidly reduce the cytochrome c. Secondly, the ΔA_{340} does arise from oxidation of glucose-6-phosphate, for addition of 6-phosphogluconate dehydrogenase results in a double yield of NADPH as the 6-phosphogluconate is enzymatically oxidized one step further. Thirdly, the addition of glutathione reductase after the reaction is complete results in disappearance of the ΔA_{340} because with the excess GSSG already present there is enzymatic reoxidation of NADPH.

Other possible reactions for producing ATP or glucose-6-phosphate have been considered. However, the necessity for adenylatekinase and hexokinase as well as AMP constitutes fairly

strong evidence that AMP functions via ADP formation with the subsequent formation of glucose-6-phosphate by the known pathway rather than some more direct transfer of phosphate to yield glucose-6-phosphate. Each alternate hypothesis requires several hypothetical reactions and all of the known requirements for glucose-6-phosphate formation are not explained.

The ATP generation reported here occurs in aqueous medium with purified biological compounds and is not dependent on submitochondrial particles, vesicles or coupling factors. It gives good yields, with little or no side reaction under optimal conditions. This phosphorylating reaction is coupled to a recently identified electron transfer which requires disulfide as a catalyst. It is clear that the GSSG, which facilitates rapid electron transfer to cytochrome c in pure solution (9) and to cytochrome c in mitochondria (11), is also involved in the energy conservation mechanism. The key intermediate would be GS· or GS^+, perhaps stabilized by GSSG.

REFERENCES

1. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Gebicki, J. M., Weinstein, J., and Schneider, A., J. Biol. Chem., 239, 614 (1964).
2. Neubert, D., and Lehninger, A. L., J. Biol. Chem., 237, 952 and 2025 (1962).
3. Weinstein, J., Schneider, A., and Hunter, F. E., Jr., Biochem. Biophys. Res. Communs., 11, 452 (1963).
4. Hunter, F. E., Jr., Scott, A., Weinstein, J., and Schneider, A., J. Biol. Chem., 239, 622 (1964).
5. Scott, A. A., and F. E. Hunter, Jr., Biochem. Biophys. Res. Communs., 11, 461 (1963).
6. Froede, H., Weinstein, J., and Hunter, F. E., Jr., Abstracts Vol. IV-50, p. 307, Sixth International Congress of Biochemistry, New York, 1964.
7. Froede, H. C., and Hunter, F. E., Jr., Federation Proceedings, 25, 647, Abstract No. 2583 (1966).
8. Hunter, F. E., Jr., Froede, H. C., and Scott, A., Proceedings of the Seventh International Congress of Biochemistry, Tokyo, Vol. V, Abstract H-93, 904 (1967).
9. Froede, H. C., and Hunter, F. E., Jr., Biochem. Biophys. Res. Communs., 38, 954 (1970).

10. Schneider, A. K., Smith, E. E., and Hunter, F. E., Jr., Biochemistry, 3, 1470 (1964).
11. Painter, A., and Hunter, F. E., Jr., Federation Proceedings, 29, #2, Abstract #3805, 914 (1970).
12. Scott, A. A., and Hunter, F. E., Jr., Biochem. Biophys. Res. Communs., 11, 461 (1963).
13. Painter, A. A., and Hunter, F. E., Jr., Science, (Submitted 1970).