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

IV. STABILITY OF INTERMEDIATES AND POSSIBLE MECHANISM OF REACTION

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.

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SUMMARY

The primary intermediates responsible for energy conservation during GSSG catalyzed electron transfer from GSH to cytochrome c under anaerobic conditions are labile but have a life time of a few minutes. The presence of phosphate results in relatively stable intermediates, presumably by forming a more stable charge transfer complex or phosphorylated form. The formation of 1 ~P per cytochrome c reduced requires postulation of a one electron transfer mechanism or a 2 electron transfer mechanism with an unrecognized acceptor for the second electron.

The intermediates in the reaction coupling phosphorylation to transfer of electrons from GSH to cytochrome c in the presence of GSSG are clearly sensitive to O_2, to true uncouplers of mitochondrial oxidative phosphorylation (2), and possibly to any free radical trap (1,2). The generation of ATP and its loss in uncoupler dependent ATPase indicate that there is an equilibrium between the intermediates in energy conservation, just as in mitochondria: Non-phosphorylated $\longleftrightarrow$ phosphorylated $\longleftrightarrow$ ATP.

The stability and nature of the intermediates in this electron transfer coupled phosphorylation are of paramount interest. In experiments in which additions of phosphate alone, ADP alone,

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and phosphate + ADP were made at timed intervals after completion of the electron transfer reaction it has been possible to learn something about the stability of the non-phosphorylated and the phosphorylated energy conservation forms under the conditions of the experiment. If they are protected from O_2 their life time is significant. This means that if they are isolatable, it will have to be under conditions and in complexes or derivatives that are stabilized against O_2. It also means that if this mechanism operates in mitochondrial energy conservation, the intermediates are in an environment or form where they are protected from O_2.

When ADP and P_i are both present during electron transfer, the enzymatic trap for measuring ATP can be added 5, 10, or 20 minutes after the cytochrome c is all reduced and the yield of ATP is similar to that when the whole system is present during electron transfer (Table I). When both P_i and ADP are delayed 5 minutes the yield of ATP is only 10-20 % of normal. When P_i is present during electron transfer and ADP added 5 or 10 minutes later the yield of ATP is nearly normal, but after 20 min the yield is down considerably. In the converse experiment, with ADP present during electron transfer and P_i added 5 or 10 minutes later, the yield of ATP is roughly half of normal. If 20 min elapse before P_i is added, there is virtually no ATP formation.

These data indicate that the primary non-phosphorylated intermediates are most unstable, although they do have a half-life which will be measurable, that phosphate probably reacts to give a much more stable intermediate, and that ADP probably reacts primarily with a phosphorylated intermediate and not with the non-phosphorylated intermediate. There is some preservation of high energy forms when ADP alone is present. This may be due to activation of ADP by the non-phosphorylated form, with later re-

TABLE I

STABILITY OF INTERMEDIATES IN THE COUPLING OF ATP FORMATION TO OXIDATION OF GSH + GSSG BY CYTOCHROME C AS REVEALED BY LATE ADDITION OF P_I, ADP, AND ENZYMATIC TRAP

ATP was measured by NADPH formation with an enzymatic trapping system in which the reaction was complete in 2 min or less in most cases. The trap consisted of hexokinase, $MgCl_2$, glucose, glucose-6-phosphate dehydrogenase (G-6-PDH), and NADP.[2] Electron transfer (cytochrome c reduction) was complete within 1 min. All cuvettes contained 45 mM Tris·HCl pH 7.6, 1 mg/ml BSA, 4.3 mM $MgCl_2$, and 1 mM EDTA. The cuvettes and solutions were flushed thoroughly with nitrogen and anaerobiosis was maintained throughout. The GSH was added to start the electron transfer reaction. After timed intervals P_i, ADP, or ADP + P_i were added. These additions were followed promptly by addition of the ATP trapping system. NADPH was measured by A_{340} in a Zeiss spectrophotometer, with experimental cuvettes balanced against an equal amount of cytochrome c as in preceding papers (1). Temp. 25°.

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 0 time 1 mM	+	+	+	+	+	+	+	+	+	+	+
Time added relative to GSH (min.):											
ADP 400 μM	0'	0'	0'	0'	5'	10'	20'	0'	0'	0'	5'
PO_4 1 mM	0'	0'	0'	0'	0'	0'	0'	5'	10'	20'	5'
Glucose at 0' 10 mM	+	-	-	-	+	+	+	+	+	+	+
Time trap added relative to GSH:	0'	5'	10'	20'	5'	10'	20'	5'	10'	20'	5'
Glucose 10 mM	-	+	+	+	-	-	-	-	-	-	-
Hexokinase 90 μg/ml	+	+	+	+	+	+	+	+	+	+	+
G-6-PDH 0.7 μg/ml	+	+	+	+	+	+	+	+	+	+	+
NADP 0.35 mM	+	+	+	+	+	+	+	+	+	+	+
Initial A_{340} x 1000 (Before ATP trap)	022	002	018	001	034	046	058	028	042	058	028
A_{340} x 1000 1 min	155	120	115	090	134	165	076	066	058	070	048
" " 2 min	159	120	120	130	135	170	098	086	094	072	055
" " 5 min	-	120	138	131	138	170	100	094	099	072	056
" " 8 min	159	121	141	131	138	170	101	096	100	072	056
ΔA_{340} x 1000	137	123	123	130	104	124	43	68	58	14	28
Calculated μM ~P	22	20	20	21	17	20	7	11	9	2	4

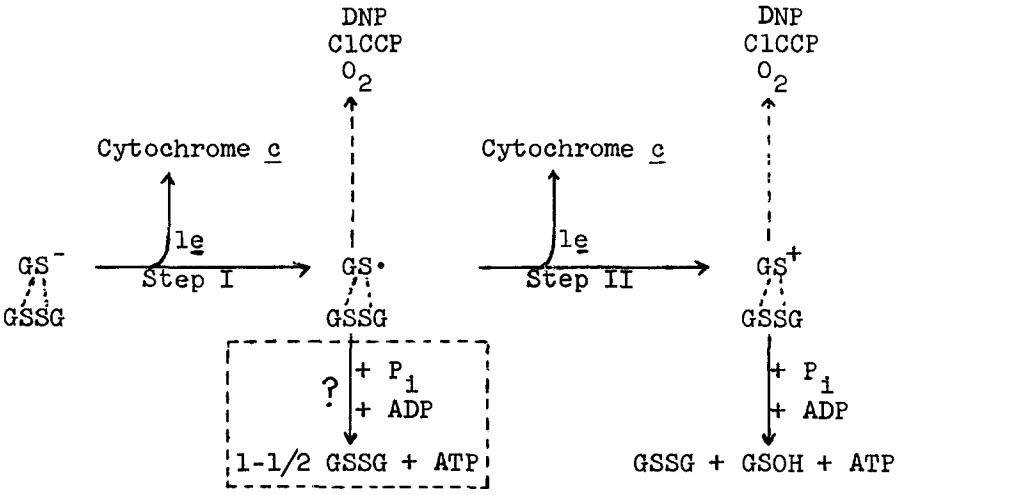
action with phosphate. It remains to be seen whether the decreased energy conservation as ATP is due to loss of non-phosphorylated intermediates or to reactions of ADP which do not lead to ATP, such as ADPPDA formation. However, all of the evidence

taken together indicates significant selectivity under the conditions of our experiments, with the preferable primary reaction being with phosphate, which is then transferred to ADP, with high yields of ATP and virtually no PP_i formation.

In proposing a possible mechanism for the coupling of phosphorylation to electron transfer from GSH to cytochrome c, the disulfide group of GSSG must occupy a key position. The metal catalyzed transfer of electrons does not result in energy conservation (1,2). For this reason the reactant GS^- and the intermediates GS· and GS^+ are shown as depending on association with GSSG for part of their properties, possibly by electron sharing in a ring of 3 sulfur atoms. The simplest scheme is presented in Fig. 1.

Oxidation of GS^- all the way to GS^+ is postulated because the formation of sulfonium ion as an energy conservation form has some basis in known high energy compounds like S-adenosyl methionine (3) and the formation of ADP and ATP from AMP and P_i during oxidation of thioethers and related compounds by Br_2 in model reactions (4-11). It has been suggested that in such reactions the electron attracting action of the sulfonium ion activates the phosphorus atom of a phosphate group for nucleophilic attack by ADP. Therefore, synthesis of ATP from ADP + P_i is shown as resulting from reaction with the sulfonium ion.

However, oxidation of GS^- to GS^+ is a 2 electron transfer, presumably occurring as indicated in 2 steps, with the thiyl radical being the intermediate, and with 2 cytochrome c molecules being reduced. Since the experiments (1) indicate a stoichiometry consistently near 1 ~P per cytochrome c reduced, there would have to be a phosphorylation coupled to both Step I and Step II removal of electrons. The mechanism for this is hard to



GSOH is of course unstable and may react with GSH ⟶ GSSG + H_2O

Fig. 1

visualize, but in Fig. 1 a second phosphorylation is indicated in the dashed box. Generation of 1 ~P would be thermodynamically possible with one electron transfer over the 500 mV oxidation-reduction potential difference from GSH to cytochrome c. Speculations involving the creation of ~P by GS· have little information to go on. With GS^+ some model reactions are known. The two steps do have in common the transfer of 1 electron from a sulfur atom to a cytochrome c.

This interpretation of the 1:1 stoichiometry would require that both GS· and GS^+ be involved in phosphorylations. However, it is possible that the reaction involves only Step I or only Step II. If GS· is the key species for phosphorylation, GS^+ may never be formed, and the reaction would involve only Step I. If GS^+ is the energy conservation species, for 1 ~P:1e stoichiometry one could visualize a mechanism in which the key reaction is a 1 e transfer from GS· to cytochrome c to yield GS^+. However, since GS^- is the starting substrate, one would also have to visualize a mechanism for GS^- to GS· that did not show in reduced cytochrome c.

The alternative to unexplained 1 ~P:1e stoichiometry would be a true value of 1 ~P:2e, with one of the electrons being accepted by compounds or groups not being measured by the A_{550} nm of cytochrome c. Some possibilities are (1) Entry of a second electron into cytochrome c, perhaps only with the GS· type reductant, to give a doubly reduced cytochrome c with unchanged A_{550}, (2) acceptance of the initial electron by GSSG to give $GS\dot{S}G$ (which might form dimers or polymerize under some conditions), (3) acceptance of the other electron by GSOH formed from GS^+ when phosphorylation occurs, (4) H_2O_2 formed from 2 GSOH ⟶ 1 GSSG + H_2O_2. A mechanism which could explain many of the properties of the system would be # 2 + # 3. In the association between GS^- and GSSG the first electron might move temporarily to GSSG to give $GS\dot{S}G$, set the stage for rapid reduction of cytochrome c by GS· and formation of GS^+, with the first electron in $GS\dot{S}G$ being transferred to the end product after GS^+ had induced phosphorylation. Regardless of whether it remained an unmeasured product or regenerated GS· (GSOH + $GS\dot{S}G$ ⟶ GS· + OH^- + GSSG) the stoichiometry for cytochrome c reduced: GS^+: ~P would be 1:1:1.

Metal ions catalyze the reduction of cytochrome c by GS^- in a reaction sequence which probably leads more directly to GSSG and not to GS^+ at all.

$$2\ GS^- + 2\ Metal^{2+} \longrightarrow 2\ GS{\cdot}Me^{1+} \xrightarrow{2e} 2\ \text{cytochrome c} \longrightarrow 1\ GSSG + 2\ Metal^{2+}$$

Energy conservation mechanisms do not exist because no GS^+ is formed, and no GSSG is present to stabilize it if it were formed.

Uncouplers and O_2 are shown as probably interrupting the sequence at the GS· intermediate to result in GSSG formation rather than GS^+ formation and phosphorylation. This could result from catalyzing dissipation of the radical form, which is normally

channeled into GS^+ formation by the GSSG. There is also the possibility that they cause rapid dissipation of the GS^+ form.

Regardless of what mechanism proves to be correct, the system must be protected against O_2 because the GS· and GS^+ forms react rapidly with O_2. In earlier work Dr. Froede looked for glutathione sulfinic and sulfonic acids. None were detected although synthetic samples were clearly separated from GSH and GSSG in the electrophoretic procedure (24). The conditions were somewhat different. However, if higher oxidation products are reduced by $\dot{GSSG}$ to give GS· for another cycle or by the excess GSH present, the net result of all oxidations could be GSSG.

For the mechanism of the phosphorylation reaction it seems logical to extend the suggestions of Higuchi and Gensch (4,5) and of Lambeth and Lardy (10), with GSSG in a sense taking the place of the Br_2 for the acceptance of one electron and formation of the first intermediate. The shift of the first electron to give $\dot{GSSG}$ would set the stage for the rapid transfer of the second electron from GS· to cytochrome c, explaining the catalysis and resulting in GS^+, partially stabilized against attack by water in a disulfide-sulfonium ion complex or ring structure. Phosphate ion would form a charge transfer complex with the stabilized sulfonium ion, resulting in a considerably more stable intermediate, be activated for nucleophilic attack by ADP through the charge transfer (electron attraction by the sulfonium sulfur), ATP would be formed, and the GSOH formed, possibly still in a ring complex with $\dot{GSSG}$, would immediately accept the extra electron to regenerate GS·, which could repeat the sequence.
Net = $1\sim P$:$1e$ to cytochrome c in a GS· $\longrightarrow$ GS^+ reaction.

In Fig. 2 the extra electron is represented as remaining in the expanded outer shell of a GSSG sulfur throughout the sequence.

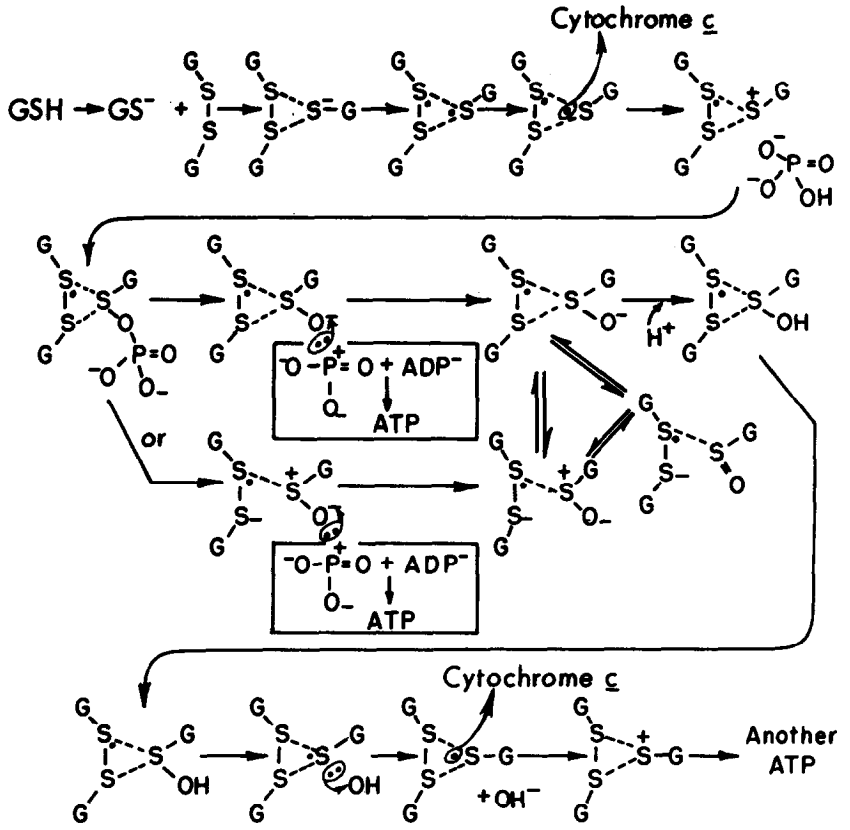


Fig. 2

It might be preferable to have it on another GSSG molecule. If a ring of 3 sulfurs is actually formed, the resonance potential and capacity for handling various numbers of electrons because of the ease of expansion of the outer shell in sulfur would be the reason the mechanism is possible. GSSG not only makes the reaction possible but also stabilizes the essential GS^+ against H_2O. The multiple sulfide and thiol groups in non-heme Fe proteins may be of special significance for this mechanism of phosphorylation. The Fe and the S may provide the separate paths for the pair of electrons. In addition to those already cited, many other workers have discussed possible roles for thiol groups in mitochondrial oxidative phosphorylation (12-23).

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