Phosphorylation Coupled to the Transfer of Electrons from Glutathione to Cytochrome c

Abstract. *Formation of adenosine diphosphate from adenosine monophosphate and inorganic phosphate can be coupled to the oxidation of reduced glutathione by cytochrome c in a reaction which requires oxidized glutathione as a catalyst. The reaction occurs with purified materials in tris(hydroxymethyl)aminomethane buffer and may represent the type reaction for one or more oxidative phosphorylations.*

The reduction of cytochrome c by reduced glutathione (GSH) requires either a trace metal ion or a disulfide, such as oxidized glutathione (GSSG), as a catalyst if it is to proceed at a significant rate (*1*). The reaction catalyzed by GSSG has properties quite different from the reaction catalyzed by metal ions and may have more biological implications. We have suggested that the reaction catalyzed by GSSG could serve as a primary step in energy conservation or oxidative phosphorylation, by means of sulfonium ion formation (*2*).

We have been able to couple the generation of a high-energy phosphate bond to the GSSG-catalyzed transfer of electrons from GSH to cytochrome c with the use of purified materials in a relatively simple buffer solution. The reaction is similar to oxidative phosphorylation in mitochondria in that it is very sensitive to uncoupling by 2,4-dinitrophenol (DNP), and the phosphorylation reaction will run in the reverse direction as if a DNP-dependent adenosine triphosphatase were present (*3*). One of the prime difficulties with reactions previously considered as a possible basis for the mechanism of oxidative phosphorylation has been their complete or relative insensitivity to DNP.

The electron transfer from GSH to cytochrome c was carried out under strictly anaerobic conditions with 40 m*M* tris(hydroxymethyl)aminomethane-hydrochloride buffer, *p*H 8.0, 1 mg of bovine serum albumin per milliliter, 10 m*M* GSSG, 1 m*M* GSH, 20 μM cytochrome c, 1 m*M* inorganic phosphate (P_i), 10 m*M* adenosine monophosphate (AMP), and 1 m*M* ethylenediaminetetraacetate. All the cytochrome c was reduced within 1 minute.

An enzymatic system to trap any adenosine diphosphate (ADP) formed was also present. This consisted of adenylkinase, hexokinase, 12 m*M* glucose, glucose 6-phosphate dehydrogenase, 5 m*M* $MgCl_2$, and 0.3 m*M* nicotinamide-adenine dinucleotide phosphate (NADP). In this enzymatic trapping system any ADP formed leads to the production of an equivalent amount of reduced NADP (NADPH). Formation of NADPH was monitored by the increase in absorption at 340 nm. The enzymes used must be free of NADPH-oxidizing enzymes, such as GSSG-reductase.

With the complete system for AMP plus P_i, about 15 μM NADPH was formed (change in absorption at 340 nm, 0.100) under the conditions described above. The reaction was completed in 2 or 3 minutes. When any one of the essential components—GSSG, GSH, phosphate, AMP, or cytochrome c —was omitted, the change in absorption at 340 nm was not significantly different from the starting value. Thus, phosphorylation of AMP was dependent on the presence of these components and the enzymatic trapping system for ADP. Formation of ATP directly from ADP and P_i was also demonstrated. In this case adenylate kinase was omitted from the enzyme trapping system; to avoid erroneous yields of ATP, one must be certain that there is no adenylate kinase in the hexokinase and glucose 6-phosphate dehydrogenase preparations used.

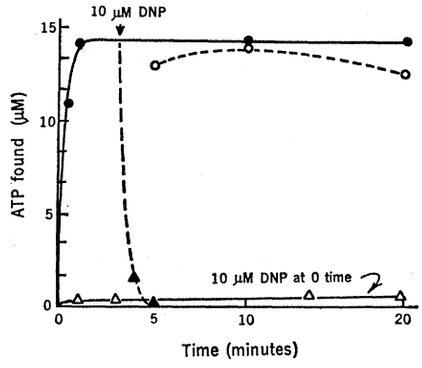

Fig. 1. Formation of ATP in the complete system with AMP + P_i and adenylate kinase, and rate of disappearance with and without the addition of 10 μM DNP. All cuvettes contained the components listed in the text. Solid circles, ATP value (ΔA_{340} readings) in the absence of DNP and with hexokinase + glucose 6-phosphate dehydrogenase present at zero time; open triangles, ATP value with 10 μM DNP and the enzyme trap present from zero time; solid triangles, 10 μM DNP added anaerobically after ATP generation was complete, and enzyme trap added 1 or 2 minutes after the DNP; open circles, ATP value when ATP was generated in the complete system, except that the trap for ATP measurement was not added until 5, 10, or 20 minutes after the reaction was started with GSH (that is, 3, 8, or 18 completed). The deviation from the control is essentially the same as the deviation from cuvette to cuvette.

With 10 μM 2,4-dinitrophenol present, no ADP or ATP formation was detected; thus complete uncoupling was observed with concentrations of DNP similar to those used to uncouple mitochondrial oxidative phosphorylation.

When ADP and ATP were generated under the standard anaerobic conditions and 10 μM DNP was added with anaerobic technique 2 minutes before the addition of the enzymatic trapping system for measuring the amount of ADP and ATP present, the ADP or ATP which was formed all disappeared in less than 2 minutes (Fig. 1). Hence the reaction probably was reversed in the presence of uncoupler to act as if an uncoupler-dependent adenosine triphosphatase were present. Similar results are observed with added ATP. The reverse reaction requires the prior generation of the same intermediate as the forward reaction. Under the conditions studied, GSSG, GSH, and cytochrome c are required. Phosphate is not an absolute requirement but does seem to stabilize the essential intermediate. The disappearance of ATP in equilibrium with the intermediate after the forward reaction is complete is very slow in the absence of uncoupler (Fig. 1). The stimulation of the rate of disappearance of ATP by 10 μM DNP is at least 100-fold.

To our knowledge this is the first electron transfer coupled phosphorylation showing the uncoupler sensitivity of oxidative phosphorylation in mitochondria. The generation of ATP by thioether and by other oxidations in anhydrous pyridine (*4*) showed some effects with 2,4-dinitrophenol at 1 to 4 molar equivalents relative to the P_i, AMP, or ADP present. These and other possible mechanisms for oxidative phosphorylation have been discussed (*5*, *6*).

The reaction mechanisms involved in the GSH–cytochrome c system must be considered as a possible prototype for energy conservation in mitochondrial oxidative phosphorylation. The thiol and the disulfide groups might be furnished by GSH and GSSG themselves. However, thiol groups and disulfides in

proteins rather than in small molecules like glutathione may provide the reactants in the case of mitochondrial phosphorylation. It is not without interest that a thiol and a disulfide have to be brought together for the system to be coupled. On the basis of this hypothesis the current concepts about conformational and configurational prerequisites for a mitochondrion to show coupled phosphorylation are not difficult to visualize as being part of the picture. The thiol and disulfide might be located in a single protein which has to assume the correct conformation, or they might be in two separate proteins which must come together. These alternatives do not exhaust the possibilities for a similar mechanism in mitochondria. The multiple sulfide-thiol relationships in the nonheme iron proteins may be of special significance for this mechanism of oxidative phosphorylation. The possible role of these proteins in energy conservation has been studied and discussed (*7*).

The reaction described here differs from model reactions with oxidation of thioethers in anhydrous pyridine (*4*, *5*) in that it uses only common biological materials, occurs in an aqueous medium, shows sensitivity to catalytic amounts of uncouplers, and can function like a DNP-dependent adenosine triphosphatase.

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8. Supported by NIH grants AM-11673-03 and HE-10107-04A.

14 April 1970; revised 2 July 1970

Transfer of Bilirubin Uridine Diphosphate–Glucuronyltransferase to Enzyme-Deficient Rats

Abstract. *Cells from a clonal strain (MH_1C_1) of rat hepatoma were transplanted subcutaneously into two homozygous Gunn rats, which are jaundiced because the enzyme bilirubin uridine diphosphate–glucuronyltransferase is absent from the liver. Because of the enzyme activity present in the transplanted cells, the recipient rats developed the capacity to conjugate bilirubin and reverted in large part to a normal pattern of bilirubin excretion.*

Of the therapeutic approaches to diseases due to enzyme deficiency, induction or transfer of enzyme activity is in many ways the most attractive. We have examined the effect of transplanting a clonal strain (MH_1C_1) of rat hepatoma cells (*1*) into homozygous Gunn rats, mutant Wistar rats which are unable to conjugate bilirubin because they lack bilirubin uridine diphosphate (UDP)–glucuronyltransferase (E.C.2.4.1.17) (*2*). The MH_1C_1 cells contain this enzyme and are capable of conjugating bilirubin in vitro (*3*).

Approximately 15 mg of MH_1C_1 cells from 10-day-old subcultures (*1*) were injected subcutaneously into the flanks of four male Gunn rats (3 weeks old). Tumors were detected in two animals about 3 weeks after transplantation. One week later the common bile duct of one of these rats was cannulated, and blood was removed from the tail for measurement of bilirubin concentration in the serum (*4*). Bile samples were collected in the dark over ice during three consecutive 12-hour intervals for measurement of bile volume and bilirubin concentration (*4*) and for analysis by thin-layer chromatography (*3*). Similar studies were performed in the second rat 8 weeks after transplantation, but in this animal pigment excretion was evaluated further with a tracer dose of [^{14}C]bilirubin and later by infusion of a bilirubin load. Normal rat serum containing 0.05 μc of unconjugated [^{14}C]bilirubin (*5*) (specific activity, 6.4 μc/μmole) was injected intravenously, and frequent bile samples were collected over the next 22 hours. Radioactivity in measured volumes of bile was assayed in a liquid scintillation spectrometer (*5*). The remaining volumes were then combined, and the fraction of the total radioactivity present as [^{14}C]bilirubin was determined by crystallization and radioassay of bilirubin (*5*, *6*) after addition of normal rat bile containing a measured quantity of nonradioactive pigment. Maximum bilirubin excretion was measured (*7*, *8*) during intravenous infusion of unconjugated bilirubin (90 μg per minute per 100 g of body weight); bile samples were collected every 10 minutes for 70 minutes, which was the duration of infusion.

Serum bilirubin concentrations in the two tumor-bearing animals were lower than those of any of five control Gunn rats that had not received MH_1C_1 cells (Table 1). Correspondingly, the biliary excretion of bilirubin in these two animals was substantially higher than that of control Gunn rats, but was lower than that of normal Sprague-Dawley rats. The absorption spectrum of diazo-

Table 1. Bilirubin excretion in normal Sprague-Dawley rats, untreated Gunn rats, and Gunn rats bearing MH_1C_1 tumors. Conjugated bilirubin (pigment I and II) was present in the bile of the normal rats and of both tumor-bearing rats but was absent from the bile of the untreated Gunn rats. The numbers in parentheses indicate the number of rats used. Values given are mean ± S.D.

Rats	Serum bilirubin (mg/100 ml)	Bile bilirubin			[^{14}C]Bilirubin excretion (% bile counts as bilirubin)	Maximum bilirubin excretion	
		Concentration (mg/100 ml)	Rate (μg hr^{-1} 100 g body weight^{-1})	Conjugated (%) *		Rate (μg min^{-1} 100 g body weight^{-1})	Conjugated (%) *
Normal (5)	0†	9.4 ± 1.6	30.5 ± 5.0	76.7 ± 16.3	77.5 ± 7.6	58.3 ± 7.4	82.1 ± 33.9
Untreated Gunn (5)	6.5 ± 1.4	0.7 ± 0.2	1.5 ± 0.9	0	20.7 ± 3.4	0.35 ± 0.2	0
Tumor-bearing Gunn rat 1	2.3	2.4	5.4	44.4			
Tumor-bearing Gunn rat 2	3.0	3.9	11.7	73.0	39.0	2.3‡	41.2

* Based on optical densities of eluted bands of conjugated and unconjugated azobilirubin. † Too low to measure. ‡ Still rising at 70 minutes.