EVIDENCE FOR DETECTION OF AT^{32}P BOUND AT THE COUPLING SITES OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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SUMMARY: A protein-bound ^{32}P-labeled substance previously detected in rat-liver mitochondria under conditions chosen to reveal possible energy-rich intermediates of oxidative phosphorylation has been identified as ^{32}P-γ-labeled ATP. The acid-precipitable protein-bound ATP (E·ATP) appears to equilibrate with medium ATP at the time of acid denaturation. After acid denaturation, the ^{32}P label of E·ATP is only slowly removed by exposure to perchloric acid containing ATP or PP_i. E·ATP is discharged in aurovertin-inhibited mitochondria during a short exposure to an uncoupler of oxidative phosphorylation in the absence of any change in the endogenous ATP pool. Under optimal energy conditions about one E·ATP is observed per two cytochrome oxidase. The results are consistent with the binding of ATP at the coupling sites of oxidative phosphorylation.

As part of the continuing search for possible phosphorylated intermediates in oxidative phosphorylation, Cross and Wang (1,2) reported that mitochondria rapidly incorporated $^{32}P_i$ into an acid-precipitable form. Characteristics of the labeling, including its prevention by oligomycin but detection in presence of aurovertin, indicated that the substance might be an intermediate in oxidative phosphorylation.

Subsequently, a similar or identical substance was detected as the difference in acid-precipitable ^{32}P in mitochondria incubated with $^{32}P_i$ with or without a short exposure to uncouplers of oxidative phosphorylation (3). Conditions resulting in solubilization of ^{32}P from the precipitate suggested that the ^{32}P might be present as a protein-bound, acyl phosphate. In the present communication, however, we report evidence that the bound ^{32}P is present as γ-labeled ATP.

MATERIALS AND METHODS

For $^{32}P_i$ purification, carrier-free $^{32}P_i$ was treated with Amberlite CG-4B,

TABLE 1

Labeling of E·ATP by $^{32}P_i$ in the Presence and Absence of Aurovertin.

Aliquots of 1 ml each of 0.25 M sucrose, 20 mM Tris, 10 mM KCl, 2 mM $MgCl_2$ (pH 7.4) containing rotenone-inhibited rat-liver mitochondria equivalent to 7.4 mg of protein were used to prepare samples and 2,4-dinitrophenol (DNP)-discharged controls as described below. Additions were made in small volumes; final concentrations were: 7.5 mM succinate, 1 mM $^{32}P_i$ (7.7×10^7 cpm), 200 μM DNP or an equal volume of water, and 0.3 M $HClO_4$, 10 mM P_i ($HClO_4$-P_i). In experiment A mitochondria were preincubated with 1.35 μg of aurovertin per mg protein. In the blank reaction, the acid-precipitable material retained 1350 cpm. The cpm listed represent averages of four replicate determinations. Error ranges represent $\pm\sigma_m$. 60 pmoles E·ATP per mg protein is observed in experiment B.

Time (seconds)	Sample A	Control A	Sample B	Control B
-90	aurovertin	aurovertin	-------	-------
0	succinate	succinate	succinate	succinate
60	$^{32}P_i$	$^{32}P_i$	$^{32}P_i$	$^{32}P_i$
70	H_2O	DNP	H_2O	DNP
74	$HClO_4$-P_i	$HClO_4$-P_i	$HClO_4$-P_i	$HClO_4$-P_i
CPM Precipitate	6170	3280	22000	4840
CPM E·ATP	2890 ± 320		17200 ± 500	

as described elsewhere (4). The pH was adjusted to 2 and carrier P_i and acid-washed activated charcoal were added. After 20 minutes, the suspension was forced through a 0.45 μ millipore filter held by a syringe adaptor, and the solution was stored at -20°. The $^{32}P_i$ solution was heated at 90° for 2 to 3 hours just prior to use. $^{32}P_i$ purified by this procedure is remarkably free from impurities that bind to mitochondrial proteins.

Unless otherwise noted, rat-liver mitochondria were labeled as described for Experiment B of Table 1. The acid-precipitable material was washed free of unreacted $^{32}P_i$ by 4 or 5 wash cycles at 0°. A wash cycle consisted of dispersion in 0.3 M $HClO_4$, 10 mM P_i followed by centrifugation at 12,000 g for 10 minutes.

RESULTS

The procedures used for detecting E·ATP are given in Table 1. The cpm listed for E·ATP represent that portion of the ^{32}P label that disappears from the acid-precipitable material during a 4 second exposure to 2,4-dinitrophenol prior to addition of perchloric acid. Conditions were chosen in these experiments to maximize our chances of detecting possible energy-rich intermediates in oxidative phosphorylation, and in each case (Sample-Control), protein-bound ^{32}P is observed which exhibits the same pH-dependent stability profile (5).

The following tests sufficed to establish that the ^{32}P label of E·ATP is due to bound ^{32}P-γ-ATP. Dissolving the acid-washed precipitate in a pH 12.4 buffer for 20 minutes at 37° followed by deproteinization results in the solubilization of approximately 90% of the ^{32}P label of E·ATP formed either in the presence or absence of aurovertin. In each case, less than 5% of the solubilized ^{32}P can be extracted with carrier P_i by isobutanol-benzene in the presence of acid molybdate. Between 80-90% of the ^{32}P label is adsorbed on activated charcoal, but after 20 minutes at 100° in 1 M HCl essentially all the ^{32}P counts can be extracted as P_i. When aliquots of the deproteinized base extract are chromatographed on an anion exchange column under conditions which allow a clear separation of P_i and ATP, the ^{32}P label showed an elution pattern like that of authentic ATP. Furthermore, within experimental error, the rate of conversion of the ^{32}P label into glucose 6-phosphate in the presence of hexokinase was identical to that of added ATP (see Table 2).

Data presented in Figures 1 and 2 indicate that bound ATP equilibrates with medium ATP during acid denaturation. The rates of appearance of $^{32}P_i$ in E·ATP and in the endogenous ATP pool are shown in Figure 1 to be identical up to and beyond the time for maximum incorporation. Furthermore, when unlabeled ATP is added at the time of acid denaturation, the amount of E·ATP observed in Figure 2 decreases linearly with decrease in the specific activity of ATP. The fact that the data give a straight line indicates that the number of binding sites for ATP remains constant over a large range of ATP concentrations.

TABLE 2

Conversion of the solubilized ^{32}P label of E·ATP into glucose 6-phosphate in the presence of hexokinase.

Hexokinase, glucose, $MgCl_2$ and carrier ATP were added to an aliquot of the deproteinized base extract and the sample incubated under appropriate conditions (6). Formation of glucose 6-phosphate was measured as the appearance of organic phosphate stable for 20 minutes in 1 N HCl at 100°. Suitable controls were run in the absence of hexokinase.

Component Measured	Conversion to glucose-6-P			
	0 time	2 min	10 min	40 min
	%	%	%	%
γ-Phosphoryl of carrier ATP	0	42	76	86
^{32}P from E·ATP	0	43	80	85

TABLE 3

Removal of the ^{32}P label of E·ATP by exposure to medium ATP and PP_i during acid-wash cycles.

Samples and 2,4-dinitrophenol-discharged controls were prepared as described. After an initial precipitation with $HClO_4$-P_i the acid-insoluble material was washed by dispersion in 0.3 M $HClO_4$, 10 mM P_i containing, where indicated, 1 mM ATP or 1 mM ATP, 10 mM PP_i. The maximum level of E·ATP is taken as that remaining after 4 wash cycles with $HClO_4$-P_i.

Wash solution	Relative level of E·ATP	
	8 wash cycles	16 wash cycles
	% of maximum level	
$HClO_4$-P_i	100	96
$HClO_4$-P_i-ATP	81	64
$HClO_4$-P_i-PP_i-ATP	37	18

Although the apparent exchange of medium ATP with E·ATP during the acid-denaturation step suggests noncovalent binding of the ATP, previous work

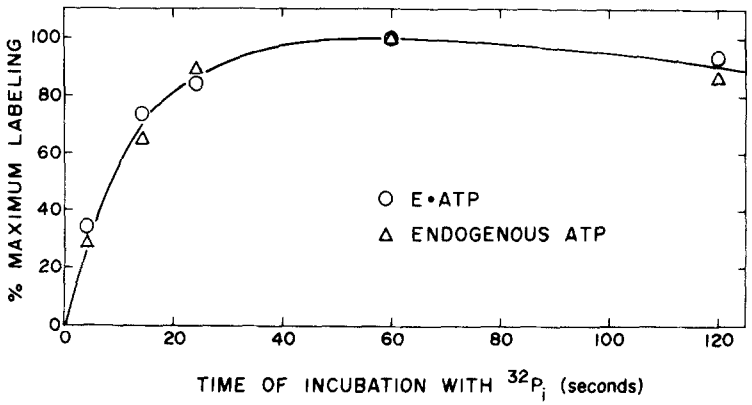

FIGURE 1

^{32}P Incorporation from $^{32}P_i$ into E·ATP and the Endogenous ATP Pool.

Samples and 2,4-dinitrophenol-discharged controls were prepared as described except that reaction was stopped as indicated after various times of incubation with $^{32}P_i$. In each case the dinitrophenol was added to the controls 4 seconds before $HClO_4$-P_i. ATP was measured as the organic phosphate discharged by the 4 second exposure to uncoupler.

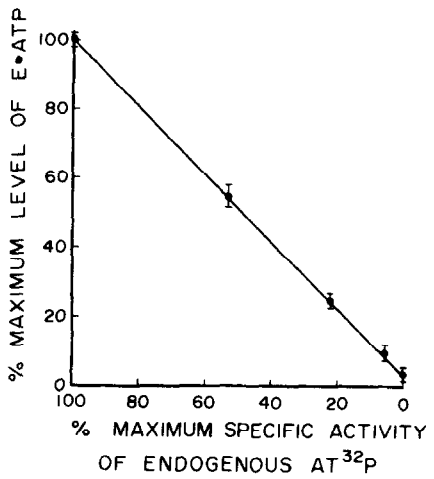

FIGURE 2

Level of E·ATP vs the Specific Activity of Endogenous ATP at the Time of Acid Denaturation

Samples and 2,4-dinitrophenol-discharged controls were prepared as described except that the $HClO_4$-P_i added to stop the reaction contained varying amounts of unlabeled ATP. Endogenous ATP was estimated to be 14 nmoles per mg of protein.

showed that the ^{32}P label remains protein bound upon dialysis against P_i, PP_i and ATP (pH 2.0) at 0° after dissolving the acid-washed precipitate in guani-

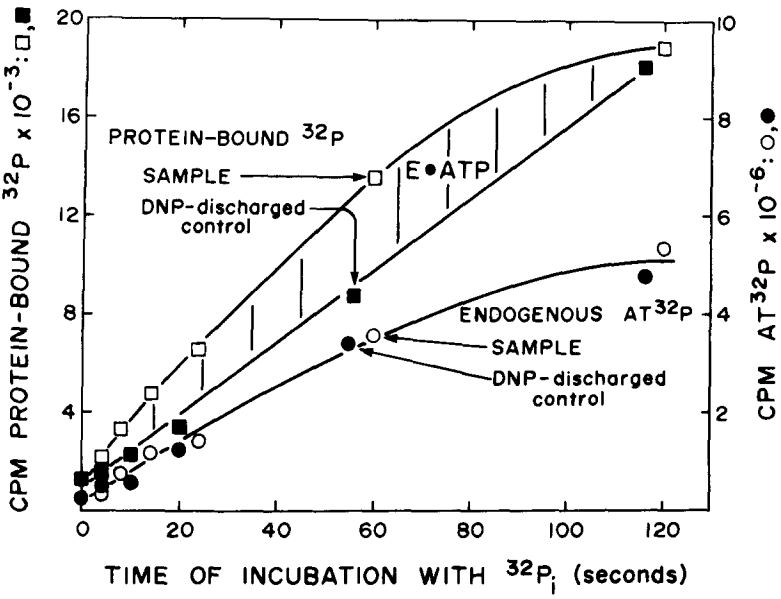

FIGURE 3

2,4-dinitrophenol-sensitive E·ATP formation in aurovertin-inhibited mitochondria which lack uncoupler-induced ATPase activity.

Aliquots of 1 ml each of the sucrose-Tris-K^+-Mg^{2+} medium containing mitochondria equivalent to 10 mg of protein were used to prepare samples and uncoupler-discharged controls, as described in Experiment A of Table 1 except that reaction was stopped, as indicated, after various times of incubation with $^{32}P_i$. In each case 2,4-dinitrophenol was added to the controls 4 seconds before $HClO_4$-P_i. The 100 nmoles of $^{32}P_i$ added contained 4.5 x 10^7 cpm. In the blank reaction the acid-precipitable material retained 474 cpm and the supernatant contained 30,000 cpm which was not P_i.

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dine-HCl or upon gel chromatography after dissolving in dimethylsulfoxide (5,7). Furthermore, the pH-stability profile and the hydroxylamine sensitivity of E·ATP are consistent with the presence of a covalent acyl-P bond (5). The experiments reported in Table 3, however, give further indication of noncovalent binding. Addition of ATP or ATP plus PP_i to the wash solution results in slow removal of the ^{32}P label of E·ATP, presumably by an exchange process.

The predictable responses of the level of E·ATP to various metabolic states and the presence of E·ATP in the membrane fraction containing the respiratory enzymes after extraction of solubile mitochondrial proteins (7),

suggest that ATP binding occurs at the coupling sites of the respiratory chain. If E·ATP represents a labeled membrane protein not associated with oxidative phosphorylation, its level might be expected to vary only in response to changes in the level of the endogenous ATP pool. This does not appear to be the case. As shown in Figure 3, E·ATP can be assayed on the basis of its sensitivity to uncouplers of oxidative phosphorylation in aurovertin-inhibited mitochondria in the absence of any uncoupler-stimulated hydrolysis of endogenous ATP. The fact that E·ATP can be discharged by a short exposure to 2,4-dinitrophenol in the absence of a corresponding change in the endogenous ATP pool suggests that E·ATP is formed on the path between the site of action of uncouplers and the ATP pool.

DISCUSSION

Procedures used in this paper are similar to but more sensitive than those which have been used successfully to detect acyl phosphate intermediates in microsomal Na^+, K^+-dependent ATPase (8,9) and in the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum vesicles (10,11). All acid-stable protein-bound ^{32}P detected in mitochondria in our experiments is accounted for as bound ATP. The limits of detection of our methodology are approximately 1 mole per 50 moles cytochrome oxidase making it unlikely that an acyl phosphate participates as an intermediate in oxidative phosphorylation. In addition, previous attempts to isolate ATP precursors in mitochondria have failed to reveal any phosphohistidine, phosphoserine or phosphorylated lipid intermediates (12). Although failure to detect a phosphorylated intermediate is by no means proof that one does not exist, the present studies provide further impetus for considering a concerted mechanism for oxidative phosphorylation in which ATP itself is the first phosphorylated product (12).

Assay of E·ATP may provide the first direct measurement of the minimum number of activated coupling sites under various metabolic conditions. Under maximum energy pressure we observe approximately one E·ATP per two cytochrome oxidase. Alternative explanations for the properties of E·ATP are, however,

possible; for example, ATP may be bound on the inner mitochondrial membrane by an enzyme of unknown function whose affinity for ATP is decreased by membrane conformational changes induced by uncouplers of oxidative phosphorylation.

Although data presented in this paper indicate to us that ATP in E·ATP is bound non-covalently, the possibility of an acyl linkage to the terminal phosphoryl of ATP cannot be conclusively eliminated. Such an intermediate has been proposed by Shamoo and Brodsky in their studies of the mechanism of Na^+, K^+-dependent ATPase (13). Were such a bond present, the mode of its formation would pose interesting questions possibly relevant to the mechanism of oxidative phosphorylation.

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REFERENCES

1. Cross, R. L., and Wang, J. H. (1970) Biochem. Biophys. Res. Commun., 38, 848.
2. Cross, R. L., Cross, B. A., and Wang, J. H. (1970) Biochem. Biophys. Res. Commun., 40, 1155.
3. Cross, R. L., and Boyer, P. D. (1971) Fed. Proc., 30, 1127.
4. Boyer, P. D., and Bieber, L. L. (1967) Methods in Enzymology, X, 768.
5. Boyer, P. D., Cross, R. L., Chude, O., Dahms, A. S., and Kanazawa, T. (1972) in "Biochemistry and Biophysics of Mitochondrial Membranes," Academic Press, New York, p. 343.
6. Darrow, R. A., and Colowick, S. P. (1962) Methods in Enzymology, V, 226.
7. Cross, R. L., and Boyer, P. D. (Academic Press, in press) in "Proc. Intern. Conf. on Mechanism in Bioenergetics," Bari, Italy, May, 1972.
8. Nagano, K., Kanazawa, T., Mizuno, N., Tashima, Y., Nakao, T., and Kakao, M. (1965) Biochem. Biophys. Res. Commun., 19, 759.
9. Hokin, L. E., Sastry, P. S., Galsworthy, P. R., and Yoda, A. (1965) Proc. Natl. Acad. Sci. U. S., 54, 177.
10. Yamamoto, T., and Tonomura, Y. (1967) J. Biochem., 62, 558.
11. Makinose, M. (1967) Pflügers Arch., 294, 82.
12. Bieber, L. L., and Boyer, P. D. (1966) J. Biol. Chem., 241, 5375.
13. Shamoo, A. E., and Brodsky, W. A. (1971) Biochim. Biophys. Acta, 241, 846.