PHOSPHORYLATION OF BOUND ADENOSINE MONOPHOSPHATE IN THE ELECTRON TRANSFER PARTICLE, DRIVEN BY SUCCINATE

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An important unsolved problem in oxidative phosphorylation is the nature of the nucleotide acceptor to which phosphate is first linked in a high energy bond. The problem has evolved to a study of the bound adenine nucleotide (1-3). It is generally accepted that ADP is the primary nucleotide acceptor of phosphate but experiments in support of this conclusion are inconclusive because it is difficult to stop the phosphorylation reaction before the primary acceptors have undergone turnover, and also because myokinase has the potentiality of redistributing the phosphoryl group among the nucleotides with great rapidity. In studying the incorporation of $^{32}P_i$ into the bound nucleotides of the electron transfer particle (ETP_H*) we have found the phosphorylation reaction to be relatively slow. Measurements of the pH changes in the presence of ETP_H have demonstrated that H^+ uptake associated with phosphorylation of bound nucleotide was completed 20 seconds after the addition of succinate at 30°. A study of the time course of the incorporation of $^{32}P_i$ into the bound nucleotides of ETP_H at 30° showed that incorporation reached its maximal value between 15 and 30 seconds after the addition of succinate. Measurements of the initial rates of phosphorylation and oxidation revealed that the initial P/O ratio was 0.2. This inefficient rate of phosphorylation in absence of exogenous nucleotide may have resulted from competition between utilization of

*ETP_H in this paper refers to a submitochondrial particle prepared from beef heart mitochondria by the method of Hansen and Smith (4). The submitochondrial particles were washed with a solution which was 0.25 M in sucrose and 0.01 M in Tris-acetate, pH 7.5, and were suspended in the same medium.

the high energy bond and dissipation of the bond by hydrolysis. The flow of chemical events leading to phosphorylation may in fact be speeded up by the experimental trapping system, i.e. by exogenous ADP, hexokinase, and glucose; in the absence of this trapping system, and by virtue of the competition mentioned above, phosphorylation of bound nucleotide apparently becomes slow enough to recognize the order in which the nucleotides are phosphorylated. A study of this phosphorylative reaction has revealed the fact that bound AMP is the first nucleotide to accept the phosphoryl group in ETP_H.

In our experiment, the electron transfer particles were preincubated at 30° as described in the legend of Table I. $^{32}P_i$ and succinate were added to the experimental flask at 2 min. The reaction was stopped 15 sec later by the addition of perchloric acid. Table I shows the amount of $^{32}P_i$ incorporated

TABLE I

INITIAL INCORPORATION OF INORGANIC $^{32}P_i$ INTO ORGANIC COMPOUNDS

Exp. no.	Preincubation period (2 minutes)		Reaction period (15 seconds)		Radioactivity (CPM/mg protein)
	$^{32}P_i$	Succinate	$^{32}P_i$	Succinate	
I	Absent	Absent	Present	Absent	2,500
			Present	Present	32,000
II	Present	Absent	Present	Absent	20,100
			Present	Present	45,000

The experimental conditions were as follows: Submitochondrial particles (ETP_H, 18 mg of protein per ml) were preincubated for 2 min at 30° in 0.9 ml of a medium which was 0.25 M in sucrose, 10 mM in Tris-acetate, pH 7.5, and 4 mM in $MgCl_2$. The reaction was initiated by the addition of undiluted $^{32}P_i$ (0.5 mC), either in presence of succinate (10 µmole) or in absence of added succinate; after an incubation period of 15 sec the reaction was terminated by the addition of perchloric acid (0.1 ml of 70% solution).

In the PCA extract, organic phosphates were separated from inorganic by the method of Lindberg and Ernster (5). The lower phase was extracted twice with water-saturated isobutanol; samples were dried, and counted in a thin window continuous gas flow counter (Nuclear Chicago).

In experiment I $^{32}P_i$ was added after the preincubation; in experiment II it was present during preincubation.

into organic phosphate by ETP_H in presence and in absence of succinate. In the absence of substrate the amount of $^{32}P_i$ incorporated into organic phosphate was only 8% of that incorporated in the presence of substrate. As a second control, the same experiment was performed with $^{32}P_i$ added at the beginning of the preincubation. In this case, the total counts incorporated were increased, but the counts incorporated in the absence of substrate were also increased some 8-fold, a figure which corresponds to the increase in the total time of incubation (9-fold).

The nucleotides extracted from the particles into acid solution were isolated collectively by absorption onto charcoal. They were separated from each other by elution from Dowex-1 according to the method of Pressman (5), and the ^{32}P content of the individual nucleotides was determined. Most of the $^{32}P_i$ incorporated into organic compounds was found to be in the ADP and ATP fractions; less than 10% of the total was found in the "flavin" and "ADX" fractions. No counts were found in the pyridine nucleotides. Table II shows the distribution of ^{32}P among the adenine nucleotides to be: AMP, 0%; ADP, 92%; ATP, 8%. These data indicate that the incorporation of P_i into the bound nucleotides of ETP_H during a short term incubation with succinate involved mainly phosphorylation of bound AMP. In the experiment in which $^{32}P_i$ was added at the beginning of the preincubation, the labeling pattern of the adenine nucleotides was different (Table II). Here the distribution of ^{32}P was: AMP, 0%; ADP, 62%; and ATP, 38%; however, the number of counts in the ADP fraction remained the same and the number in the ATP fraction increased by about 6.5 fold, a figure which again approximates the increased time of incubation with $^{32}P_i$ (9 fold). This experiment demonstrates that the incorporation of $^{32}P_i$ into ATP is a slow, time-dependent reaction which is independent of added substrate and could involve a mechanism such as ATP-P_i exchange. The fact that the radioactivity incorporated into ATP in the absence of added substrate was approximately proportional to time also indicates that bound ATP was not hydrolyzed by ATPase.

TABLE II

THE DISTRIBUTION OF ^{32}P AMONG THE BOUND ADENINE NUCLEOTIDES OF ETP_H FOLLOWING INCUBATION WITH SUCCINATE FOR 15 SECONDS

Exp. no.	Preincubation period (2 minutes) $^{32}P_i$	Preincubation period (2 minutes) Succinate	Reaction period (15 seconds) $^{32}P_i$	Reaction period (15 seconds) Succinate		CPM/mg protein	Percentage of total
I.	Absent	Absent	Present	Present	Total	26,900	100
					AMP	0	0
					ADP	24,700	92
					ATP	2,200	8
II.	Present	Absent	Present	Present	Total	37,200	100
					AMP	0	0
					ADP	22,900	62
					ATP	14,300	38

In general, the experimental conditions were similar to those described in the legend of Table I, the final volume of the reaction mixture in this case being 9 ml. The nucleotides in the perchloric acid extracts were adsorbed onto charcoal, the $^{32}P_i$ being washed out with 1% perchloric acid. The nucleotides were eluted from charcoal with a solution which was 50% in ethanol and 2% in ammonia; the eluate was evaporated to dryness. The nucleotides were separated from each other on a Dowex-1 (formate) column under the conditions described by Pressman (5).

In Experiment I the $^{32}P_i$ was added, together with succinate, after the 2 min preincubation period.

In Experiment II the $^{32}P_i$ was present during the preincubation.

Myokinase activity could account for the incorporation of counts into ADP. Myokinase having free access to all three nucleotides (ADP and ATP being the only radioactive species) should bring about the labeling of ATP in both the α and β positions. Double labeling of ATP would also result if AMP were first phosphorylated with $^{32}P_i$ and the ADP so formed were further phosphorylated. We have degraded the radioactive ATP formed during 15 seconds of incubation by reacting it with D-3-phosphoglycerate in the presence of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase. The conversion of ATP to ADP was determined quantitatively by measurement of the decrease in the absorbancy of DPNH at 340 mμ. The samples were then deproteinized by the addition of perchloric acid and the nucleotides were separated from each other by ion exchange chromatography. The fact that the ADP isolated from the reaction mixture was practically free of radioactivity (Table III) shows that the ^{32}P in the ATP fraction was in the terminal position almost exclusively. This experiment indicates that the bound nucleotides were not freely equilibrated by myokinase during the 15 seconds of incubation.

As shown in Table II, increase in $^{32}P_i$ incorporated into ATP after prolonged

TABLE III

REDISTRIBUTION OF RADIOACTIVITY AFTER CLEAVAGE OF THE TERMINAL PHOSPHORYL GROUP OF ATP

	ATP (mμmoles)	ADP (mμmoles)	Total radioactivity in nucleotides (CPM)
Before reaction	100	0	78,000
After reaction	0	89	2,600

The experimental conditions were similar to those described in Table I. The labeled ATP was separated from the perchloric acid extract and was diluted with unlabeled ATP.

The terminal phosphate was hydrolyzed by reaction with 3-phosphoglycerate in the presence of phosphoglycerate kinase and glyceraldehyde dehydrogenase (Boehringer ATP test kit). The conversion of ATP to ADP was determined quantitatively by measurement of the change in absorbancy (of DPNH) at 340 mμ. After the hydrolysis, the reaction mixture was stopped by perchloric acid (final in concentration). The supernatant was charged on Dowex-1-formate column after neutralization with KOH. The nucleotides were separated as described in Table II.

incubation with $^{32}P_i$ (Exp. II) is not paralleled by an increase in $^{32}P_i$ incorporation into ADP. This result suggests that under our experimental conditions myokinase does not act in the direction of ATP + AMP ⟶ 2 ADP. Siekevitz and Potter (7) have reported that the removal of ATP by the hexokinase-glucose trap increases the rate at which mitochondrial myokinase activity converts ADP to ATP + AMP. It was also reported by Hansen and Smith (4) that the presence of an ATP trapping system such as hexokinase-glucose increases the P/O ratio from 0.2 to 1.0. We can assume, then, on the basis of these various considerations that an internal myokinase can act in the direction of 2 ADP ⟶ ATP + AMP when the ATP trapping system is present.

Since ADP contained 92% of the total $^{32}P_i$ incorporated into the bound adenine nucleotides at the initial stage of phosphorylation, and since the incorporation of $^{32}P_i$ into ADP appeared not to be due to myokinase activity, it follows that ADP must have been formed by a direct phosphorylation of AMP. We conclude, therefore, that AMP is the primary acceptor nucleotide for high energy phosphate and that ADP formed by this transfer is converted to ATP by an internal myokinase in the presence of the ATP trapping system.

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