RESPONSE OF AN UNIDENTIFIED PHOSPHATE FRACTION AND OF BOUND PHOSPHOHISTIDINE IN MITOCHONDRIA TO Ca^{++}, UNCOUPLERS, AND INHIBITORS*

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Our interest in the protein-bound phosphohistidine of mitochondria prompted studies on its responsiveness to respiratory chain inhibitors, to uncouplers of oxidative phosphorylation, and to a transient energy load as imposed by active transport of Ca^{++}. Experiments with Ca^{++} were stimulated by the preliminary report of Pressman (1963) that Ca^{++} caused an apparent rise in the level of bound phosphohistidine in mitochondria. These studies were underway at the same time that alternative assay procedures for bound phosphohistidine were being developed, and more attention was being given to the use of "tightly-coupled" mitochondria for metabolic studies. As work progressed, it became clear that at least one ^{32}P component in addition to phosphohistidine appeared in column eluates with assay conditions as originally used to detect bound phosphohistidine (Suelter et al., 1961).

Data presented in this paper demonstrate that the increase in apparent protein-bound ^{32}P induced by Ca^{++} and by K^+ and valinomycin (Pressman, 1964), represents an increase in an unidentified phosphate fraction accompanied by a decline in phosphohistidine level. In addition, information is reported on the marked sensitivity of the unidentified fraction to uncouplers and respirat chain inhibitors.

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Detection of the unidentified fraction and its response to Ca^{++} - When mitochondria are incubated for short periods with $^{32}P_i$, then dispersed in 7.5 M urea-0.3 M NH_4OH and passed through an ion exchange column, all the $^{32}P_i$ and $AT^{32}P$ is retained but most of the protein and lipid together with any bound ^{32}P pass into the eluate. When aliquots of such eluates were mixed with phenol, a characteristic partitioning of the radioactivity was observed to occur. Results of a representative experiment, and the effect of Ca^{++} are shown in Table I. Phenol extracts nearly all the protein from the diluted

TABLE I

PRESENCE OF ^{32}P-PHOSPHOHISTIDINE AND AN UNIDENTIFIED ^{32}P-FRACTION IN COLUMN ELUATES

INCUBATION CONDITIONS	^{32}P AS BOUND PHOSPHOHISTIDINE	^{32}P IN UNIDENTIFIED FRACTION
	%	%
No Ca^{++}	0.123	0.050
10^{-3} M Ca^{++}	0.060	0.313
Control	0.000	0.008

Mitochondria (rat liver, 10-15 mg. protein) were incubated with 2.0 ml of solution containing 2.0 x 10^{-3} M $MgCl_2$, 1 x 10^{-5} M phosphate (approximately 3 x 10^6 cpm $^{32}P_i$) 0.20 M sucrose, and 0.05 M Tris-Cl, pH 7.2 for 1 minute at 30° C, then 6.0 ml of 7.5 M urea-0.3 M NH_4OH was added. $^{32}P_i$ was added to the control immediately after addition of the urea-NH_3. 5.0 ml aliquots of the dispersion were passed through a 10 x 0.5 cm Dowex-1-hydroxide (X-200) column followed by 8.0 ml of H_2O. A 1.0 ml aliquot of the column eluate was mixed with 2.0 ml of 88% phenol. After 30 seconds, 4.0 ml of 0.01 M KH_2PO_4-0.01 M EDTA, pH 7.6, was added and the solution thoroughly agitated and centrifuged. The radioactivity extracted into phenol was taken as a measure of bound phosphohistidine and that remaining in the aqueous layer as the unidentified fraction.

column eluates. Over 95% of the ^{32}P-protein extracted by the phenol from short-time incubations of mitochondria with $^{32}P_i$ has acid lability characteristics of phosphohistidine. Identity of the ^{32}P component in such fractions with phosphohistidine has also been demonstrated by alkaline digestion and co-chromatography with synthetic phosphohistidine as described previously (DeLuca et al., 1963). The material remaining in the aqueous layer, unlike phosphohistidine, behaves like P_i as measured by formation of a molybdate complex soluble in isobutanol-benzene assay (see Lindberg and Ernster, 1955).

Measurements of the amount of bound phosphohistidine present at differe time intervals with and without delayed addition of Ca^{++} gave results shown in Fig. 1. In the absence of Ca^{++}, the phosphohistidine level rapidly reach a maximum (see also Bieber et al., 1964). Addition of 10^{-3} M Ca^{++} at 30 sec results in a slower decline of phosphohistidine to about one half of the

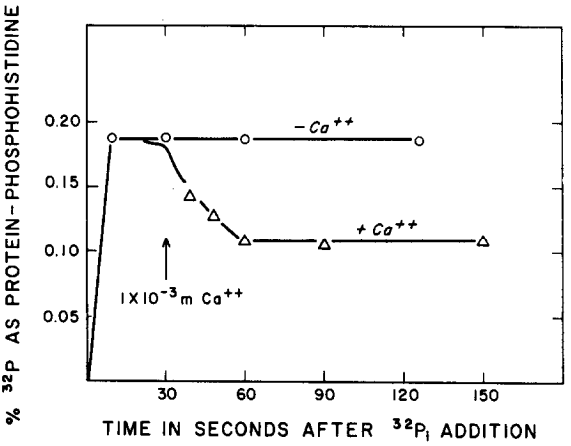

Figure 1. Depression of bound phosphohistidine labeling by Ca^{++} additi The incubation medium was identical to that described in Table 1. The mitochondria (10-15 mg protein per sample) were incubated at 30° for 1 minute pri to the addition of high specific activity $^{32}P_i$ (approximately 5 x 10^6 cpm). Calcium was added to some samples 30 seconds after addition of the $^{32}P_i$. The incubation was terminated by the addition of 8.0 ml of cold 0.3 M trichloroacetic acid. After centrifugation in the cold, the protein pellet was dissolved in 1.0 ml of 7.5 M urea-0.3 M NH_4OH, 2.0 ml of 88% phenol were added, and after 45-60 minutes, the phenol layer was washed repeatedly with 0.1 M KH_2PO_4—0.01 M EDTA, pH 7.6, until no $^{32}P_i$ could be removed from the phenol layer. The protein was next precipitated from the phenol by addition of 8.0 ml of acetone. After mixing the precipitated protein with successive additions of $CHCl_3$, MeOH, and ether 5:1:6, v/v, centrifugation and methanol wash, the protein was dispersed in 2.0 ml of 0.10 M NaOH—0.01 KH_2PO_4.

After reprecipitation of the protein with 3.0 ml of cold 0.6 M trichlorc acetic acid, 2.0 ml of 0.3 M trichloroacetic acid—0.01 M KH_2PO_4 were added and the samples placed in a boiling water bath for 1 minute, chilled for 2 minutes and centrifuged in the cold. The radioactivity released during the 1 minute hydrolysis was taken as a measure of the bound phosphohistidine.

previous level. Data in Fig. 2 show the time course of appearance of ^{32}P int the phosphohistidine plus the unidentified phosphate fraction and into the ATP. The labeling in presence of Ca^{++} occurs rapidly. The subsequent declir over the 4 minute period likely represents decrease in the phosphohistidine component. The Ca^{++} markedly depresses the labeling of the ATP. Thus the

increase in ^{32}P in the column eluates would appear to arise from the $^{32}P_i$ and not from any $AT^{32}P$ formed.

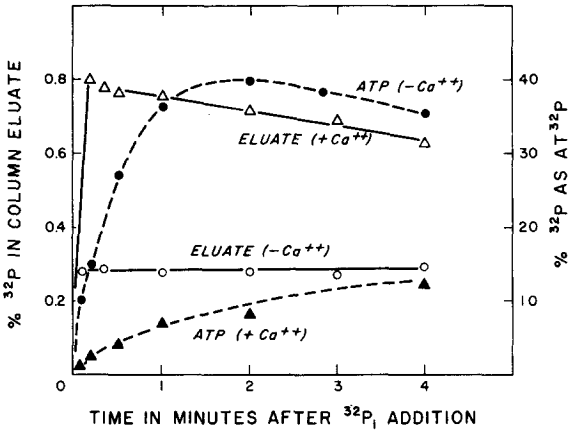

Figure 2. Stimulation of protein-bound ^{32}P labeling by Ca^{++} addition. The incubation medium and assay conditions are identical to that described in Table 1. The protein was precipitated from 1.0 ml aliquots of the 7.5 M urea-0.3 M NH_4OH solution by addition of 4.0 ml of 0.3 M trichloroacetic acid and P_i extracted from the deproteinized supernatant as the isobutanol-benzene soluble molybdate complex (see Lindberg and Ernster, 1955). $AT^{32}P$ was taken to be that radioactivity remaining in the lower layer.

The response of the unidentified phosphate fraction to Ca^{++} infers, in harmony with suggestions of Pressman (1964), that the component is related to active transport. Possibly this or a similar component may be related to transport systems involved with K^+ and Na^+. Pressman (1964) has shown definitive effects of K^+ on his apparent protein-bound fraction, and Charnock and Post (1963) and Albers et al., (1963) have demonstrated a labile protein phosphate associated with K^+ and Na^+ activated ATP-ase.

Effects of inhibitors and uncouplers - Data presented in Table II show that 2,4-dinitrophenol and to a greater extent m-Cl-carbonylcyanide phenylhydrazone (m-Cl-CCP) have a pronounced inhibitory effect on the labeling of the unidentified fraction after a 10 second incubation in the absence of calcium. This suggests that the appearance of ^{32}P in the fraction is dependent upon the primary coupling process(es) of oxidative phosphorylation. The moderate stimulation of the labeling of bound phosphohistidine with a short incubation

TABLE II

EFFECT OF INHIBITORS AND UNCOUPLERS ON THE 10 SECOND LABELING OF BOUND PHOSPHOHISTIDINE AND A LABILE UNIDENTIFIED FRACTION OF MITOCHONDRIA

INHIBITOR	DISTRIBUTION OF ^{32}P AS % OF CONTROL[a]		
	BOUND PHOSPHOHISTIDINE	UNIDENTIFIED FRACTION	ATP
None (control)	100	100	100
Dinitrophenol, 2×10^{-5} M	127	100	65
" , 4×10^{-4} M	120	50	48
m-Cl-CCP, 4×10^{-7} M	127	62	87
" , 1×10^{-5} M	132	49	52
Oligomycin, 5 μg/ml	104	100	57
Antimycin, 1 μg/ml	110	34	47
Rotenone, 3×10^{-7} M	110	100	45

a. Control showed 6.5% ^{32}P as ATP, 0.011% as bound phosphohistidine, and 0.012% in the unidentified fraction.

The rat liver mitochondria (2.2 mg per sample) were brought to room temperature in 0.25 M sucrose and then were incubated at 30° C in 2.0 ml of an incubation medium (identical to that described for Table 1) with the inhibitors for 10 seconds prior to the addition of high specific activity $^{32}P_i$ (approximately 12 x 10^6 cpm). All inhibitors except dinitrophenol were added to the incubation medium in 5 μl of 95% ethanol. After 10 secs. incubation with $^{32}P_i$, the reaction was terminated by addition of 6.0 ml of 7.5 M urea-0.3 M NH_4OH. $AT^{32}P$ was taken to be the radioactivity remaining in the lower layer of the phosphomolybdate extraction assay (see Lindberg and Ernster, 1955) as assayed with a deproteinized aliquot of the 7.5 M urea-0.3 M NH_4OH solution.

as compared to the inhibitory effects of dinitrophenol and m-Cl-CCP on the phosphohistidine labeling with longer incubation as reported previously (Suelter et al., 1961) suggest a complex interplay of factors involved in the maintenance of the phosphohistidine labeling. It has been reported that the readily soluble fraction of mitochondria capable of forming bound phosphohistidine is closely associated with succinate thiokinase (Mitchell et al., 196 Kreil and Boyer, 1964). Oligomycin did not appreciably inhibit the labeling of either the unidentified fraction or the phosphohistidine although markedly depressing ATP labeling. This suggests that the ^{32}P present in both fractions arises from $^{32}P_i$ and not $AT^{32}P$. The pronounced decrease in labeling of the unidentified phosphate fraction in presence of antimycin but not of rotenone remains to be adequately explained.

Chemical nature of the unidentified fraction - The chemical nature of the ^{32}P-labeled material appearing in the column eluates is unknown. The assay procedure is consistent with but does not establish a protein binding. It appears that the inhibitors and calcium are acting upon different unidentified phosphate components because antimycin A and m-Cl-CCP do not prevent Ca^{++} stimulation of the rise in ^{32}P in the unidentified fraction, but inhibit ^{32}P appearance in absence of Ca^{++}. Chromatography of column eluates from both calcium treated and nontreated samples on Sephadex G-25 results in appearance of most of the ^{32}P with the low-molecular weight substances. However, the labeling in the unidentified fraction is retained with the protein upon ultrafiltration of column eluates. Such findings suggest that the ^{32}P might be present as specifically occluded P_i or as an exceptionally-labile but covalently-bound phosphoryl group.

The method for detection of the unidentified component(s) by the column assay is not entirely satisfactory. In particular the stopping of the ^{32}P labeling by the addition of the 7.5 M urea-0.3 M NH_4OH is not instantaneous. There is a small lag so that addition of $^{32}P_i$ with the urea-NH_3 has been observed to give partial labeling of the unknown fraction. Also a weak labeling of ATP and bound phosphohistidine from $^{32}P_i$ continues for a short period after urea-NH_3 addition. The very sensitive assay and the probability that less than one turnover at an active site may give labeling puts a severe test on stopping conditions not encountered in usual enzymic assays. The definite possibility must be considered that the component(s) is an experimental artifact. However, the response of the component(s) to a low concentration of metabolic inhibitors and to various ions as shown by us and by Pressman (1964) points to a metabolic function.

Summary - Short exposure of mitochondria to Ca^{++} results in a decrease in labeling of bound phosphohistidine from $^{32}P_i$, but an increase in an unidentified phosphate fraction. In the absence of Ca^{++}, the labeling of this fraction is inhibited by short exposure to low concentrations of

2,4-dinitrophenol or m-Cl-carbonylcyanide phenylhydrazone but not by oligomycin. The unidentified ^{32}P fraction contains one or more components which appear to represent protein-bound, exceptionally-labile phosphoryl derivative(s) or specifically occluded inorganic phosphate.

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