# RESPIRATION-DEPENDENT FORMATION OF ACID-STABLE AND ACID-LABILE FORMS OF MITOCHONDRIAL PHOSPHOPROTEINS AND THEIR POSSIBLE INTERCONVERSION

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Reports from several laboratories have established that $P^{32}$-labeled mitochondrial phosphoprotein is formed during respiration in the presence of $P_i^{32}$ or $ATP^{32}$, and have discussed a possible role of phosphoprotein as an intermediate in oxidative phosphorylation or some other respiration linked mitochondrial function (Friedkin and Lehninger, 1949; Kennedy and Smith, 1954; Judah, 1961; Boyer et al., 1962).

Two types of labeling of mitochondrial phosphoprotein have been observed. In one, the protein-bound $P^{32}$ is present in an acid-stable form which can be isolated as serine phosphate after acid hydrolysis (Kennedy and Smith, 1954). The second contains $P^{32}$ in an acid-labile, base-stable form which can be isolated as phosphohistidine after alkaline hydrolysis of the protein (Boyer, et al., 1962).

In this paper new information on the chemical and biochemical properties of mitochondrial phosphoprotein will be presented which suggests that both acid-labile and acid-stable phosphate groups may be related components in transphosphorylation reactions linked to respiration. The data in Table I confirm that intact rat liver mitochondria as well as submitochondrial particles prepared by the digitonin method of Devlin and Lehninger (1958) rapidly incorporate labeled inorganic phosphate during respiration in the absence of ADP into a form that is nondialyzable after disruption of the membrane structure by the alkaline-urea procedure of Boyer, et al., (1962). This incorporation is inhibited by agents such as dinitrophenol that uncouple oxidative phosphorylation and by respiratory inhibitors. Homogenization of the dialyzed fractions with chloroform-methanol

extracted less than 3% of the radioactivity showing that only a very minor fraction of the $P^{32}$ incorporated could be accounted for as mitochondrial phospholipid. Between 75-90% of the protein-bound $P^{32}$ has been found to be present in an acid-labile form, recovered as indicated in Table 1. The remainder, designated as Fraction B, is stable to repeated extraction with 5% trichloroacetic acid at room temperature and gives rise to serine phosphate after hydrolysis in 6 N HCl for 12 hours.

Table 1

Formation of Acid-Labile and Acid-Stable Phosphoprotein

Each reaction system contained 0.125 mM potassium phosphate ($4 \times 10^6$ cpm), 12.5 mM β-hydroxybutyrate, 0.02 M Tris buffer pH 7.4, 2 mM $MgCl_2$, 0.1 M sucrose and 50 mg mitochondrial protein in Experiment 1 and 10 mg protein of digitonin particles in Experiment 2. Final volume was 4.0 ml. Mitochondria or digitonin particles added to initiate reaction at 30° for 3 minutes. Reaction was terminated by rapid addition of 10 ml of alkaline urea (8 M urea-1% ammonium carbonate, pH 9.0). Protein-bound phosphate was separated from reaction system by dialysis against 2 changes of 0.05 M Tris buffer, pH 8.0 at 2° for 30 hours (Fraction A). Identical results have been obtained by separation of inorganic phosphate and nucleotides from protein-bound $P^{32}$ on Sephadex G-25 by eluting with the urea-ammonium carbonate pH=8.5. Acid-stable phosphoprotein was determinec as the protein-bound radioactivity remaining after washing protein 3 times with 7% trichloroacetic acid at room temperature (Fraction B). The difference in the radioactivity between Fraction A and Fraction B is ascribed to acid-labile phosphoprotein.

| Experiment | Addition | CPM in Phosphoprotein Acid Labile | Acid-Stable |
|---|---|---|---|
| Intact mitochondria | None | 28,300 | 11,100 |
| | Dinitrophenol (0.5 mM) | 5,200 | 2,200 |
| | Sodium cyanide (1.0 mM) | 3,100 | 1,000 |
| | Boiled mitochondria | 200 | 100 |
| Digitonin particles | None | 10,200 | 3,000 |
| | Dinitrophenol (0.5 mM) | 2,500 | 800 |
| | Boiled particles | 200 | 200 |

Rate and equilibrium studies (Fig. 1) show that incorporation of $P^{32}$ into the acid-labile form reaches a plateau level prior to the plateau level of the acid-stable form. Subsequent addition of unlabeled inorganic phosphate to the

system, with further incubation, results in dilution of the label in the two forms of phosphoprotein, as well as a similar sequential approach to new plateau or steady-state levels of protein-bound $P^{32}$ showing the reversibility of the incorporation reactions. Both the rate of approach to the plateau level and the magnitude of net phosphate incorporated are obviously functions of the inorganic phosphate concentration.

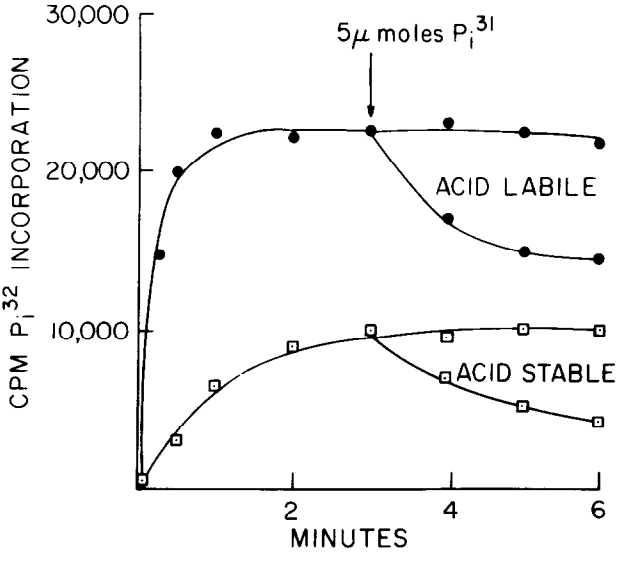


Figure 1

Rate of Incorporation of Inorganic Phosphate into Mitochondrial Protein. The reaction system and analytical details are the same as described for Table 1.

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One interpretation of these results is that inorganic phosphate and the two forms of mitochondrial phosphoprotein are components of an equilibrium system in which the acid-stable form accepts phosphate from the acid-labile form possibly by way of an N→O transphosphorylation reaction. Experiments described in Table 2 show that there is a rapid conversion of acid-stable P to acid-labile P when the acid-stable form of mitochondrial phosphoprotein is exposed to base. Incubation at pH 10.5 converts over 60% of the acid-stable form to an acid-labile form in 1 hour. Evidence that the structure of the protein moiety is critical

Table 2

Conversion of Acid-Stable to Acid-Labile Phosphoprotein

The pH of 1.0 ml aliquots of Fraction B (Table 1) containing 1400 cpm per ml was adjusted to pH 10.5 and incubated for the indicated time at 35° in a final volume of 2.0 ml. At the end of the incubation half the reaction system was mixed with trichloroacetic acid at 15% final concentration and centrifuged, the precipitate washed 2 times with 2 ml 5% TCA, suspended in 0.1 M $NH_4OH$, plated, and the radioactivity determined. The remainder of the incubation system was adjusted to pH 7.5 and dialyzed for 24 hours against 2 changes of 0.1 M tris-HCl buffer, pH 7.5, plated and counted. Trypsin treated-Fraction B used in Experiment 2 was prepared by incubation of 5 mg crystalline trypsin (Worthington) with 120 mg protein of Fraction B for 30 minutes at pH 8.0, 30°. A control sample was treated in identical manner except that trypsin was omitted. The results are presented as the difference in radioactivity (cpm) between the sample dialyzed at pH 7.5 and the corresponding TCA treated sample obtained following incubation at pH 10.5 for the indicated times. Each result is the average of triplicate samples.

| Experiment | Treatment | $-\Delta$CPM $P^{32}$ |
|---|---|---|
| 1 | Fraction B-1 minute | 0 |
| | Fraction B-30 minutes | 240 |
| | Fraction B-60 minutes | 470 |
| 2 | Fraction B-60 minutes | 390 |
| | Fraction B-Trypsin-60 minutes | 20 |
| | Fraction B-Trypsin-90 minutes | 30 |

in the overall conversion was given by the finding that a trypsin-treated acid-stable fraction does not yield the acid-labile form when incubated at pH 10.5 (Table 2). Fractionation of the trypsin digest by paper electrophoresis at pH 6.4 in pyridine-acetic acid-water (10:0.4:90) followed by paper chromatography in n-butanol-acetic acid-pyridine-water (5:1:3:4) resulted in isolation of a peptide containing 80% of the radioactivity of the original acid-stable fraction. This peptide yielded serine phosphate and free serine after hydrolysis in 6N HCl but no detectable amount of histidine (i.e. less than 0.5% of the total serine found) when analyzed by the automatic ion exchange chromatographic method of Spackman, et al., (1958). The absence of histidine, which Boyer (1962) has shown to be responsible for the acid-labile nature of mitochondrial phosphoprotein might explain the inactivity of the trypsin-treated protein in the acid-stable $\rightarrow$ acid-labile transformation.

Studies of the effect of pH on the transformation of the acid-stable form to acid-labile form indicates dependence on an ionized species having a pK' of approximately 8.5 and is therefore consistent with observations of Bergmann, et al., (1923) on O→N acyl migrations. Furthermore, the results are strikingly similar to those reported by Taborsky and Allende (1962) from studies of a reversible intramolecular transphosphorylation reaction by phosvitin.

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