METABOLIC FUNCTION OF PHOSPHOHISTIDINE

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The quest for chemical intermediates of oxidative phosphorylation led Boyer and coworkers to the isolation and identification of phosphohistidine from a mitochondrial protein digest (Boyer et al, 1962). The rapid dynamic equilibrium between the phosphohistidine yielding protein fraction and both P_i^{32} and ATP^{32} were deemed appropriate for an intermediate of oxidative phosphorylation. The failure of DNP at concentrations amply sufficient to completely uncouple oxidative phosphorylation systems (3×10^{-5} - 10^{-4} M) to prevent the formation of protein bound phosphate presented some difficulty and was interpreted in terms of the specialized status of the mitochondrially bound adenine nucleotides and phosphate (Suelter et al, 1961). This view appeared inconsistent to us with the observations of Chance and Hollunger (1963) that the intramitochondrial energy transfer components, as evidenced from the spectrophotometric behavior of the electron carriers, do respond to the same levels of uncouplers as complete phosphorylation systems.

In contrast to the procedure employed by the Boyer group for observing the effects of inhibitors on the rate of incorporation of P_i^{32} into protein and nucleotides, our experimental technique has been to permit P^{32} equilibration of these components prior to the addition of the inhibitor. Changes in labeling of the appropriate fractions thus represent primarily changes in net amounts of components rather than alterations of their P_i^{32} equilibration rates. In Fig. 1 we observe that 2×10^{-5} M dinitrophenol (DNP) has no difficulty in effecting a drop in total fixed phosphate. (Analysis by ion exchange chromatography shows

that prior to the DNP addition >85 per cent of the total fixed P^{32} is ATP.) Protein bound phosphate unexpectedly underwent a slight but significant rise.

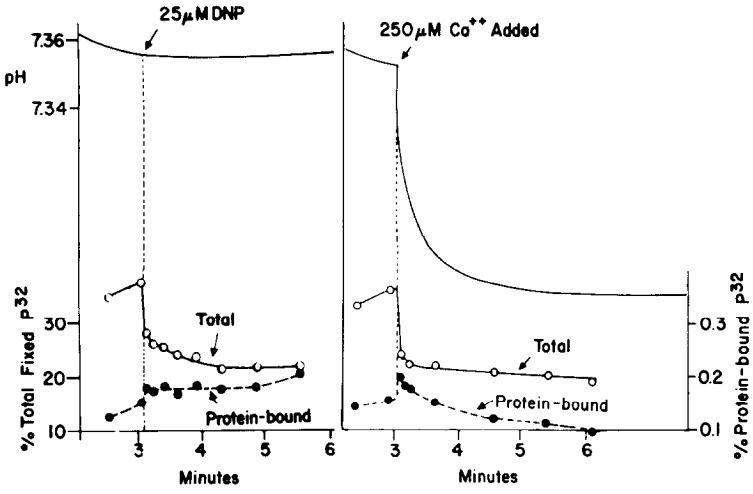

Fig. 1 (left). Effect of DNP on Protein-bound P^{32}. The temperature stabilized reaction vessel (19.6°) was equipped with a magnetic stirrer and standard Beckman glass and calomel electrodes attached to a recording pH meter (Δ.1 pH = 5"). The incubation medium consisted of: 10 mM TRIS-Cl, pH 7.4; 25 mM KCl, 3.3 mM $MgCl_2$, 200 mM sucrose, 10 μm phosphate containing 16 x 10^8 c.p.m. P^{32}; final volume, 8 ml. Moist O_2 was blown across the surface of the incubation mixture and, following additions of rat liver mitochondria (50 mg protein equivalent) (procedure of Schneider. 1948) and appropriate inhibitors, .5 ml samples were removed with a rapid sampling syringe and ejected into 3 ml chilled urea (40 per cent)-triton X-100 (4 per cent)-ammonia (.3 M). Net fixed phosphate was determined on a .1 ml aliquot of the digest by the Berenbaum and Chain (1938) procedure and the protein bound P^{32} determined by passing the remainder through a 3 x .5 cm Dowex-1-formate (X-10) column, followed with a .5 ml triton-ammonia-urea wash (cf. Suelter et al (1961)).

Fig. 2 (right). Effect of Ca^{++} on Protein-bound P^{32}. Conditions same as Fig. 1.

It was thought that the response of protein bound phosphate to perturbations of the mitochondrial energy transfer processes would be even more informative if a transient energy load could be imposed. Chance (1956) reported that Ca^{++} can produce just such an effect as evidenced by transient alterations of mitochondrial respiration and electron carrier status. It has also been established that the active transport of Ca^{++} into mitochondria is characterized by

the expulsion of H^+ (Saris, 1959) and hence the time course of the resultant energy load can be followed with a pH meter (Saris, 1963).

In Fig. 2 a slight but significant transient rise in protein bound phosphate correlates with the expulsion of H^+ from the mitochondria and the rapid fall of total fixed phosphate. A variable but always discernible rise in this fraction occurs at low Ca^{++} concentrations.

We have previously reported that the antibiotic valinomycin, which has an uncoupler like action (McMurray and Begg, 1959) has certain properties which differentiate it from other uncoupling agents (Pressman, 1963). When low levels of this agent are added to the basic system a dramatic elevation of protein bound phosphate ensues (Fig. 3) accompanied, as in the case of Ca^{++} (Fig. 2), with a drop in the extramitochondrial pH and a decrease of total fixed phosphate. This effect is more consistent than the Ca^{++} effect reported, and often leads to as much as a three-fold rise in protein bound phosphate.

Oligomycin, which prevents the discharge of fixed phosphate by valinomycin, effectively blocks ATP from the locus of valinomycin action (Fig. 4). Since the rise in protein bound P^{32} produced by valinomycin is entirely unaffected by this block, we conclude that the phosphate moiety of protein bound phosphate need not arise from ATP but can be derived directly from P_i.

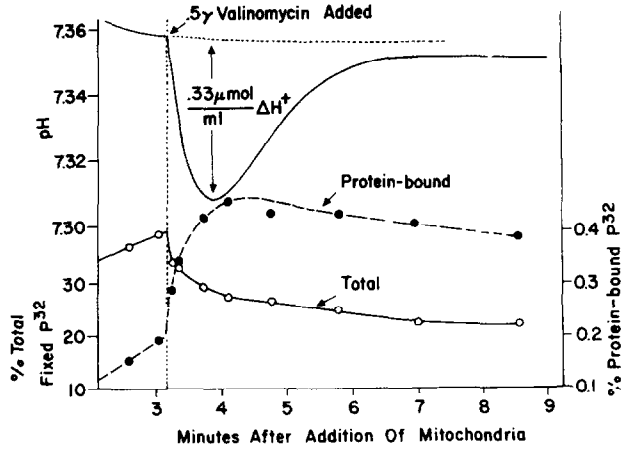

Fig. 3. Effect of Valinomycin on Protein-bound P^{32}. Conditions same as Fig. 1; 0.33 μmol ΔH^+ = 53 μmol H^+/gm mitochondrial protein.

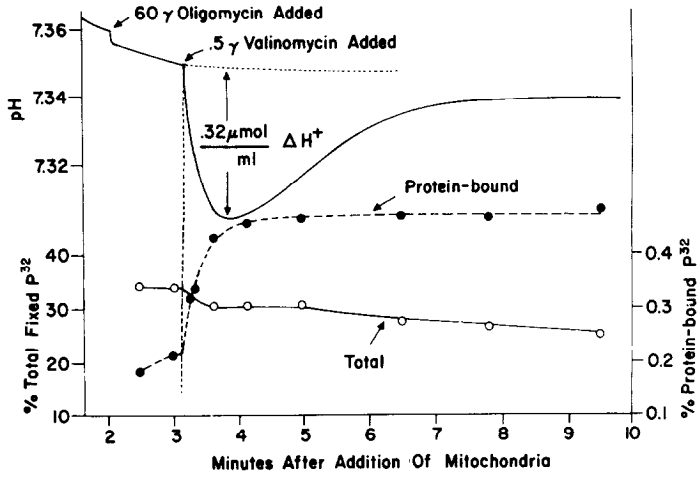

Fig. 4. Effect of Oligomycin on Valinomycin Induced Protein-bound P^{32} Rise. Conditions same as Fig. 1.

Although we prefer to refer to the P^{32} labeled protein as "protein bound phosphate," our presumptive evidence is that this is for the most part the "bound phosphohistidine" described by Boyer et al (1962). Acid labile phosphate was determined routinely on all bound protein fractions and varied between 65 and 100 per cent, the higher figures obtaining during shorter incubation periods. Only a relatively small portion of the acid stable protein bound phosphate could be protein-phosphoserine by the criterion of alkali lability under the conditions of Ahmed and Judah (1963). The acid lability assays displayed considerably more variability than the total protein bound phosphate values and accordingly the latter were chosen for the experimental figures presented here.

If the steady state of a sequence of consecutive reactants is altered by removal of a terminal product, it follows, providing no complicating feedback loops exist, that the levels of all precursors of the product decrease. In our experiments, low levels of DNP or Ca^{++} and all levels of valinomycin tested cause a dephosphorylation of mitochondrially bound ATP. The concomitant rise in protein bound phosphate can thus be taken as strong evidence against its functioning as an intermediate on the main pathway of ATP formation.

Recent work in our laboratory has established that nanogram quantities of valinomycin activate a powerful mitochondrial potassium pump, the pH decrease

following the addition of the antibiotic correlating with the active transport of K^+ into the mitochondria (Moore and Pressman, 1964). Thus figures 3 and 4 relate the evaluation of protein bound phosphate with the active transport of K^+. Upon being pumped into mitochondria, Ca^{++} possibly induces detrimental side effects which partially obscure the participation of protein bound phosphate in ion transport. K^+ is presumably a more physiological substrate for the mitochondrial ion pump and, upon stimulation of its transport, the relationship of protein bound phosphate to ion transport is revealed more clearly. Our conclusion, therefore, is that protein bound phosphate, including phosphohistidine, plays a role in supplying energy for the active transport of ions.

The failure of oligomycin to prevent the formation of protein bound phosphate, which in turn is stimulated by active transport, suggests a close parallel with the observations of Ahmed and Judah (1962) on the increase of protein-phosphoserine labeling with P^{32} during ion transport of Na^+ in liver slices.

Although we would not rule out a participation of both protein-phosphohistidine and protein-phosphoserine in active transport, the observation that the protein bound phosphate initially formed (first 20 seconds) is virtually 100 per cent acid labile, mitigates against the possibility that protein-phosphohistidine arises artifactually from protein-phosphoserine as suggested by Wadkins (1963).

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