THE ASSOCIATION OF READILY-SOLUBLE BOUND PHOSPHOHISTIDINE FROM MITOCHONDRIA WITH SUCCINATE THIOKINASE*

R. A. Mitchell, L. G. Butler†, and P. D. Boyer

Department of Chemistry, Division of Biochemistry
University of California
Los Angeles, California

Received June 17, 1964

Phosphohistidine, probably bound as an integral part of a polypeptide chain, has been shown to be closely linked to mitochondrial phosphorylations (Boyer et al., 1962; DeLuca et al., 1963). A soluble extract from bovine liver mitochondria has been obtained in which bound phosphohistidine can serve as an intermediate between P_i and ATP (Peter et al., 1963), and this phosphohistidine has been considered as a possible intermediate in oxidative phosphorylation (Boyer, 1963). More recent results as outlined in this paper demonstrate, however, a close association of the readily soluble form of bound phosphohistidine from mitochondria with succinate thiokinase. A similar association also has been found with highly purified E. coli succinate thiokinase (Kreil and Boyer, 1964).

Preliminary evidence of relations between phosphohistidine and succinate thiokinase - Several observations made in the course of study of the soluble mitochondrial extract capable of forming bound phosphohistidine from $^{32}P_i$ or $AT^{32}P$ suggested possible participation of low-molecular weight organic components in the labeling. These were (a) considerable variability in capacity and rate of labeling in different extracts, (b) decrease in rates of discharge of ^{32}P from bound phosphohistidine in partially purified protein

* Supported in part by a grant from the National Institute of General Medical Sciences of the U. S. Public Health Service

† National Science Foundation Predoctoral Fellow

fractions[1] after dialysis or charcoal or Sephadex treatment even though sufficient Mg^{++} or Mn^{++} was added, (c) acceleration of discharge rates by the supernatant solution from boiled and centrifuged soluble mitochondrial extracts and (d) the failure of the ash of the boiled extract to accelerate discharge. Such observations led to testing of effects of various known cofactors on the discharge. DPN promoted ^{32}P release somewhat, but coenzyme A was found to be particularly effective. ADP was considerably less effective than GDP. GDP alone or succinate together with coenzyme A rapidly and completely discharged the bound ^{32}P. Fumarate and α-ketoglutarate had much less effect than succinate, and chlorosuccinate, malate, malonate, citrate, oxalate, mercaptosuccinate and glutarate were without effect.

These results suggested participation of succinate thiokinase in the formation of bound phosphohistidine. Further support to this suggestion was given by the demonstration of a competitive relationship between succinate and phosphate, and the promotion of the phosphorylation of the histidine residue by succinyl coenzyme A, as shown by the data of Fig. 1 which represents the equilibrium levels of bound phosphohistidine plotted as a function of P_i concentration. Such data permit the maximum amount of phosphorylated intermediate to be found by extrapolation. The effects of succinyl coenzyme A or succinate plus coenzyme A addition on this maximum value are shown in Table 1.

Puzzling but interesting peculiarities of the discharge of ^{32}P from bound phosphohistidine have been encountered in other experiments. Thus the response of bound phosphohistidine to various reagents may be influenced by the prior treatment of the soluble extract (Table 2). These results suggest possible relationships in the crude extract with other enzyme activities and

---

[1]Supernatant fractions obtained by sonicating frozen and thawed bovine liver mitochondria were purified by organic solvent precipitation, then exposed to high specific activity $^{32}P_i$ in the presence of 2 mM Mg^{++} or Mn^{++} for 1 minute before addition of excess EDTA to stop the reaction. Further purification by column chromatography was done in the presence of EDTA to prevent loss of protein-bound ^{32}P.

TABLE I

GENERATION OF CAPACITY FOR BOUND PHOSPHOHISTIDINE FORMATION BY EXPOSURE TO SUCCINYL COENZYME A

TREATMENT PRIOR TO SEPHADEX SEPARATION	ADDITIONS FOR INCUBATION WITH $^{32}P_i$	MAXIMUM PHOSPHOHISTIDINE FORMED
		M x 10^{-8}
None	0.05 mM CoA	4
None	1 mM succinate	4
None	0.05 mM CoA 1 mM succinate	0.4
0.05 mM CoA 1 mM succinate	0.05 mM CoA	2
0.05 mM CoA 1 mM succinate	1 mM succinate	2
0.05 mM CoA 1 mM succinate	0.1 mM succinyl CoA	8

The experiment was performed as described in the legend to Fig. 1

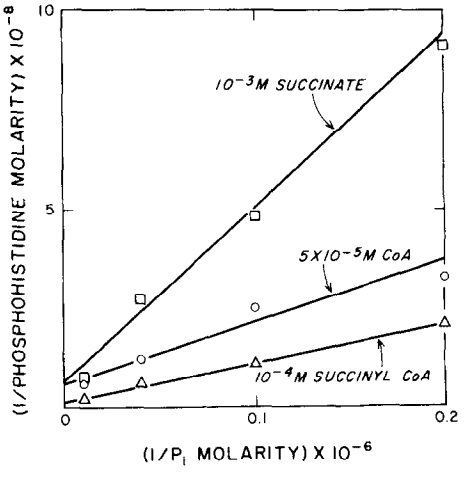

Figure 1. A 100,000 xg. supernatant fraction from sonicated bovine liver mitochondria was eluted from a Sephadex G25 column with a solution containing 5 mM $MgCl_2$, 0.1 mM P_i at pH 7.4. After stirring the eluate with Dowex-1 (acetate) and charcoal, the mixture was filtered and 2 ml. aliquots of the filtrate (containing approximately 2 mg. protein) were incubated for 2 minutes with $^{32}P_i$, 7.5 mM tris acetate, pH 7.5, and varying levels of potassium phosphate (5 x 10^{-6} M, 10^{-5} M, 2.5 x 10^{-5} M, 10^{-4} M) in the presence of 10^{-3} M succinate, 5 x 10^{-5} M CoA, or 10^{-4} M succinyl CoA. Bound phosphohistidine was determined by a phenol extraction procedure (Bieber et al., 1964).

TABLE 2

CHANGES IN PHOSPHOHISTIDINE LABELING RESULTING FROM DOWEX-1 ACETATE TREATMENT OF A SOLUBLE MITOCHONDRIAL EXTRACT

ADDITIONS TO ASSAY SYSTEM	BOUND PHOSPHOHISTIDINE FORMED	
	CHARCOAL TREATED	CHARCOAL AND DOWEX-1 ACETATE TREATED*
	M x 10^8	M x 10^8
None	2.62	1.33
1 mM hydroxylamine	0.99	2.01
1 mM succinate	1.59	2.56
0.1 mM CoA	1.35	2.11
1 mM succinate + 1 mM hydroxylamine	1.17	1.23
0.1 mM CoA + 1 mM hydroxylamine	0.98	3.36

* Readjusted to 0.1 mM in potassium phosphate following removal of Dowex-1 acetate. Incubations were made with the additions shown and as described for Fig. 1.

interconversion of "high-energy" forms in the preparation.

Succinate thiokinase appears to account adequately for the bound phosphohistidine readily liberated in soluble form from mitochondria. Some phosphohistidine not associated with succinate thiokinase appears to be formed during oxidation of β-hydroxybutyrate by aged rat liver mitochondria depleted of succinate thiokinase by swelling in 0.033 M P_i buffer at pH 7.4 (Lindberg and Duffy, 1964). It would, indeed, be somewhat surprising if the phosphorylate imidazole group were unique to succinate thiokinase.

Labeling patterns with partially purified succinate thiokinase from bovine liver - A correlation was noted between capacity for phosphohistidine formation and succinate thiokinase activity (Kaufman et al., 1953) in fractions from mitochondrial extracts. The rate of formation of ^{32}P-labeled bound phosphohistidine from 0.1 mM $^{32}P_i$ in a 20-fold purified fraction (approximately 1 μmole of succinyl hydroxamate formed per min. per mg. protein at 37°) was enhanced by succinate, coenzyme A, and succinyl coenzyme A, listed in order of increasing effectiveness. However, only in the presence of all substrates was the labeling of the phosphohistidine sufficiently rapid to warrant its consideration as a phosphoenzyme intermediate. Fig. 2 shows the rate of appearance

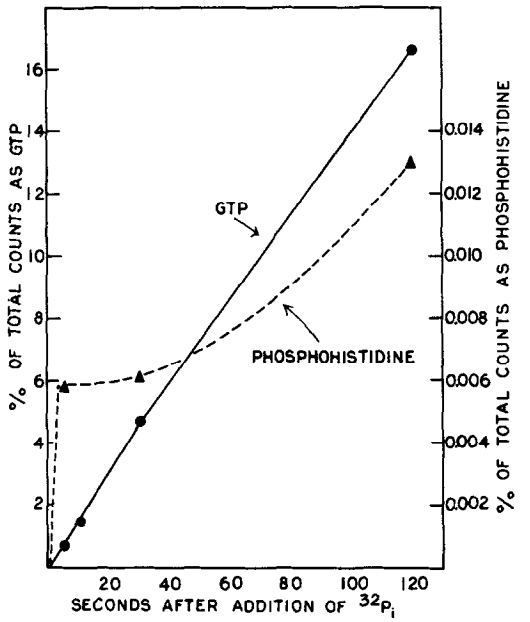

Figure 2. The incubation mix contained in 0.5 ml final volume 0.1 mM succinate, 0.1 mM coenzyme A, 0.1 mM P_i, 0.04 mM succinyl CoA (Simon, 1957), 1.0 mM GTP, 1.0 mM GDP, 5.0 mM $MgCl_2$, 200 mM Tris acetate, pH 7.4, and 1.1 mg. succinate thiokinase purified 20-fold from bovine liver mitochondria. After 120 seconds incubation at 37° one drop of high specific activity P_i^{32} was added and the reaction was stopped at various time intervals by addition of 0.2 ml of 0.5 M EDTA, pH 8. Protein-bound phosphohistidine was determined by a phenol extraction procedure and GTP^{32} was determined on an aliquot of the aqueous extracts by a modification of the procedure of Berenblum and Chain (1938).

of ^{32}P in GTP and phosphohistidine in the presence of near equilibrium mixture of all substrates. At 120 seconds over 16% of the ^{32}P is in GTP; at isotopic equilibrium, approximately 90% of the ^{32}P would be expected in the GTP. The initial rapid jump in phosphohistidine implies a rapid equilibration with $^{32}P_i$. The continued rise likely reflects an even more rapid equilibration of the phosphoryl group on histidine with GTP, and the increase in specific activity of the GTP. The specific activity of the bound phosphohistidine would be determined by the relative rates of equilibration with the P_i and GTP pools and the specific activity of these pools.

These findings are consistent with the phosphorylated imidazole group serving as an intermediate in the formation of GTP from P_i by bovine liver succinate thiokinase. Other studies (Kreil and Boyer, 1964) implicate bound

phosphohistidine as the unidentified phosphorylated form of E. coli succinate thiokinase (Upper, 1964).

Conclusion - Protein-bound phosphohistidine has been found to be closely associated with succinate thiokinase in bovine liver mitochondrial extracts. Present data are consistent with the phosphorylated imidazole group serving as an intermediate in the formation of nucleotide triphosphate from P_i by succinate thiokinase. Bound phosphohistidine may serve as a valuable parameter for mechanism studies on the exchange patterns and other aspects of the thiokinase reaction (Hager, 1962; Upper, 1964; Cha and Parks, 1964).

REFERENCES

Berenblum, I., and Chain, E., Biochem. J., 32, 295 (1938)
Bieber, L. L., Lindberg, O., Duffy, J. J., and Boyer, P. D., Nature, in press (1964)
Boyer, P. D., Science, 141, 1147 (1963)
Boyer, P. D., DeLuca, M., Ebner, K. E., Hultquist, D. E., and Peter, J. B., J. Biol. Chem., 237, PC3306 (1962)
Cha, S., and Parks, R. E., J. Biol. Chem., in press (1964)
DeLuca, M., Ebner, K. E., Hultquist, D. E., Kreil, G., Peter, J. B., Moyer, R. W., and Boyer, P. D., Biochem. Z., 338, 512 (1963)
Hager, L. P., in The Enzymes, Boyer, P. D., Lardy, H., and Myrback, K., Eds., Vol. 6, p.387, Academic Press, New York (1962)
Kaufman, S., Gilvarg, C., Cori, O., and Ochoa, S., J. Biol. Chem., 203, 869 (1953)
Kreil, G., and Boyer, P. D., Biochem. and Biophys. Res. Comm., accompanying publication (1964)
Lindberg, O., and Duffy, J. J., Unpublished data (1964)
Peter, J. B., Hultquist, D. E., DeLuca, M., Kreil, G., and Boyer, P. D., J. Biol. Chem., 238, PC1182 (1963)
Simon, E., in Biochemical Preparations, Vol.5., Shemin, D., Ed., p.30, John Wiley and Sons, Inc., New York (1957)
Upper, C. D., Ph.D. Dissertation, University of Illinois, Urbana (1964)