DETECTION OF BOUND PHOSPHOHISTIDINE IN E. COLI SUCCINATE THIOKINASE*

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A phosphoryl enzyme has been considered as a possible intermediate in the reaction catalyzed by succinate thiokinase (see Kaufman, 1955; and review by Hager, 1962). Recently, Upper (1964) demonstrated the uptake of ^{32}P into E. coli succinate thiokinase after incubation with $AT^{32}P$. Release of the label from the ^{32}P-enzyme could be accomplished with ADP or coenzyme A.

In the preceding communication (Mitchell et al., 1964), experiments were described, which indicated that a protein-bound phosphohistidine is closely associated with succinate thiokinase partially purified from bovine liver mitochondria. In this paper the detection of bound phosphohistidine in highly purified E. coli succinate thiokinase is described. This finding together with the observed labeling pattern suggest that phosphohistidine is an intermediate in the succinate thiokinase reaction.

Labeling and degradation of the enzyme - Succinate thiokinase was purified from E. coli grown aerobically with succinate as the sole carbon source by the method described by Upper (1964). An enzyme preparation containing about 35 units[1] per mg of protein was used for most of the experiments described below.

Labeling of the enzyme was achieved by incubation with succinyl coenzyme A (prepared according to Simon, 1957) and $^{32}P_i$. The phosphoprotein was extracted

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[1] 1 unit = 1 μmole succinyl hydroxamate per minute at 37°. Assay mixture (Knight, 1961): 0.1 M Tris (pH 7.2), 0.016 M mercaptoethanol, 0.02 M $MgCl_2$, 0.2 M disodium succinate, 0.015 M ATP, 0.7 M NH_2OH, 0.2 mM coenzyme A.

into phenol and repeatedly washed with 0.01 M phosphate buffer (pH 7.6), until no further radioactivity could be removed. After addition of bovine serum albumin as carrier, the protein was precipitated with acetone, washed with acetone and dried in vacuum. The phosphoprotein was stable to alkali but quite labile to acid; exposure to 0.3 M trichloroacetic acid for 1 minute at 100° liberated all radioactivity as $^{32}P_i$. Chromatographic separation of an alkaline hydrolysate of the phosphoprotein gave the pattern shown in Figure 1.

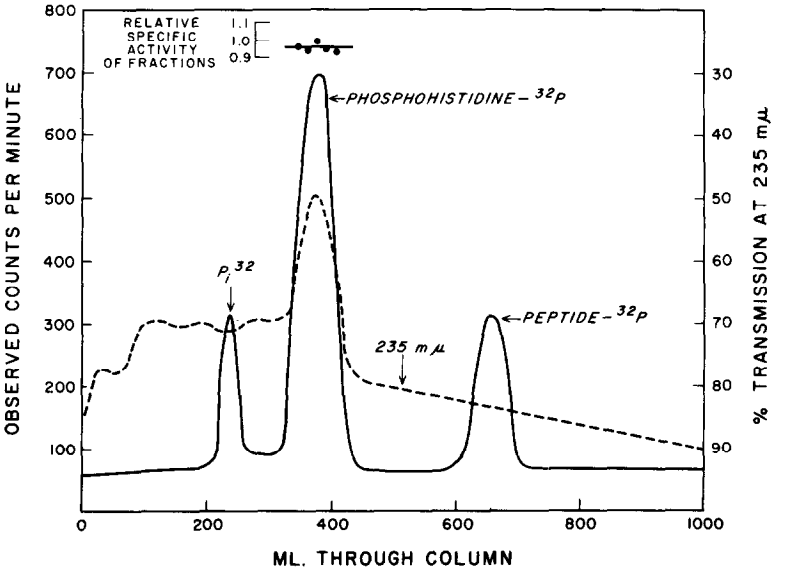

Figure 1. Co-chromatography of synthetic phosphohistidine and an alkaline digest of ^{32}P-labeled E. coli succinate thiokinase.

A partially purified and ^{32}P-labeled E. coli succinate thiokinase (8 units per mg.) was digested with 20 mgs. of synthetic phosphohistidine in 3 N NaOH for 80 minutes at 100-105° in a sealed tube. The sample was chromatographed as described previously (DeLuca et al., 1963), and the % transmittance at 235 mμ and ^{32}P measured in the eluate flowing from the column by continuous recording. Specific activity of peak tubes was determined by acid hydrolysis and measurement of P_i and ^{32}P liberated.

The principal radioactive component co-migrated with added synthetic phosphohistidine and the specific radioactivity was constant throughout the peak. In this experiment, a second more slowly migrating compound was observed, which was probably a peptide containing phosphohistidine similar to that found during identification of phosphohistidine from beef liver extracts. The amount $^{32}P_i$ liberated during the hydrolysis in 3 N NaOH corresponded closely to the extent of cleavage of the added synthetic phosphohistidine. The radioactivity present in the phosphohistidine peak and the probable peptide peak was converted to P_i by exposure to 1.5 N H_2SO_4 for 20 minutes at 30°. In an experiment similar to that reported in Fig. 1, but with a highly purified preparation (35 units per mg) and with a longer hydrolysis time (140 instead of 80 minutes) only the ^{32}P-phosphohistidine peak was observed.

These experiments clearly identify the ^{32}P component in the E. coli succinate thiokinase preparation as bound phosphohistidine. The chromatographic procedure used will separate the two phosphohistidine isomers, and the product from E. coli corresponds to the same isomer as that from liver and heart mitochondria. Identification of whether the biological product is the 1- or 3-phosphoryl histidine is not yet completed.

Although the results make it quite probable that the phosphorylated histidine residue is a part of the catalytically active succinate thiokinase, the homogeneity of any succinate thiokinase preparation has not been established by rigorous criteria. Thus a close association of two or more proteins in the preparation remains a possibility.

Effects of substrates on the labeling of the enzyme - The effect of various substrates on the amount of bound phosphohistidine present after incubation with $^{32}P_i$ for 1 minute is shown in Table 1.

As noted in experiment 1, incubation with succinyl-coenzyme A and $^{32}P_i$ gave considerable labeling of the protein. The level of bound phosphohistidine was approximately doubled by increasing the phosphate concentration from 0.5 to 2.0 mM. A tenfold excess of ADP over succinyl-coenzyme A effectively depleted

TABLE 1

EFFECT OF SUBSTRATES ON LABELING OF E. COLI SUCCINATE THIOKINASE

EXPERIMENT	ADDITIONS (MILLIMOLARITY)	MOLES BOUND ^{32}P PER 10^5g PROTEIN
1	0.5 P_i; 0.2 succinyl CoA	0.59
	2.0 P_i; 0.2 succinyl CoA	1.27
	2.0 P_i; 0.2 succinyl CoA; 2.0 ADP	0.06
2	0.5 P_i	0.03
	0.5 P_i; 0.1 CoA	0.51
	0.5 P_i; 0.1 CoA; 4.0 succinate	0.02
	0.5 P_i; 0.1 CoA; 4.0 ADP	0.08
3	0.5 P_i; 0.1 ATP	0.01
	0.5 P_i; 0.1 ATP; 0.1 CoA	0.25
	0.5 P_i; 0.1 ATP; 0.1 succinate	0.01
4	0.5 P_i; 0.1 succinate: 1 min.	0.03
	4 min.	0.09
	15 min.	0.29
	0.5 P_i; 2.0 succinate: 4 min.	0.32

Incubations were made for 1 minute (if not otherwise indicated) at 37° with 0.1 M Tris buffer (pH 7.4), 0.01 M $MgCl_2$, and $^{32}P_i$ (equivalent to $\sim 10^7$ c.p.m.) in a final volume of 0.5 ml. The reaction was stopped by addition of 1 ml of liquefied phenol.

the amount of phosphoprotein. Experiment 2 shows the somewhat surprising finding that uptake of $^{32}P_i$ into phosphohistidine also occurred upon incubation with added $^{32}P_i$ and coenzyme A. This indicates that the enzyme is isolated in a phosphorylated or some other activated form. Labeling from $^{32}P_i$ in the presence of ATP required coenzyme A, while ATP alone or ATP plus succinate did not promote any significant ^{32}P uptake (experiment 3). As shown in experiment 4, a very slow labeling of the enzyme occurred in the presence of succinate alone and the rate of this labeling increases with increasing succinate concentration.

The requirement of coenzyme A for a rapid labeling of succinate thiokinase from $^{32}P_i$ suggests that a covalently bound coenzyme A may participate in the

reaction sequence. This possibility has been stressed by Upper (Upper, 1964) and is also in harmony with kinetic data (Cha and Parks, 1964). The very slow labeling in the presence of succinate is difficult to reconcile, however, with a compulsory binding of coenzyme A prior to the release of phosphate from bound phosphohistidine. Clearly, much more information on the rate and extent of phosphohistidine formation in the succinate thiokinase system will be needed to understand the exchange reactions and to establish a reaction mechanism.

Conclusion - Bound phosphohistidine has been identified in highly-purified E. coli succinate thiokinase. The enzyme preparation as isolated is in an activated or phosphorylated form, and can be labeled from $^{32}P_i$ upon incubation with coenzyme A, succinyl coenzyme A, or, much more slowly, with succinate. The findings are consistent with but do not establish bound phosphohistidine as an intermediate in the succinate thiokinase reaction.

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