ENZYMATIC PHOSPHORYLATION OF SERINE BY INORGANIC PYROPHOSPHATE

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Received October 8, 1968

O-phospho-L-serine is a well recognized compound in intermediary metabolism. No enzymes which cause the phosphorylation of free serine have been described thus far. Thus the compound is produced in the course of serine biosynthesis by transamination from O-phosphohydroxypyruvate (see Pizer, 1963). The formation of O-phospho-L-serine by the action of O-phospho-L-serine phosphatase entails phosphate exchange between the pre-formed substance and free serine (Neuhaus and Byrne, 1959, 1960; Borkenhagen and Kennedy, 1959; Bridgers, 1965; Pizer, 1964), and not net synthesis. The phosphorylation of protein-bound serine by a variety of substances has, in contrast, been observed repeatedly. Thus the serine at the active site of phosphoglucomutase may be phosphorylated by glucose-1,6-diphosphate (see Najjar, 1962), that of phosphorylase by ATP (Fisher *et al.*, 1959) and that of alkaline phosphatase by orthophosphate (see for instance Schwartz, 1963). We wish to report the discovery of an enzyme in *Propionibacterium shermanii* which catalyzes the phosphorylation of free L-serine to O-phospho-L-serine by means of inorganic pyrophosphate:

$$\text{L-serine} + PP_i \longrightarrow \text{O-phospho-L-serine} + P_i.$$

P. shermanii (ATCC 9614) was grown as described before (Friedmann, 1968). The bacteria were stored at -22°. Cell-free extracts were made by grinding with glass beads and water in an ice-cooled Sorvall Omni-Mixer (Friedmann, 1968). After dilution with twice the volume of water origi-

nally added, the suspension was centrifuged for 20 min at 12.000 x g. The clear supernatant solution, containing about 10 mg protein per ml, was treated with one fifth volume of 0.7% protamine sulfate solution (Elanco Products Company), and the precipitate was removed by centrifugation. The supernatant solution (pH about 7.5) was brought to a pH of 4.8 by careful addition of 0.5 N acetic acid, and gently stirred at 0° for 15 min. After removal of the brown precipitate by centrifugation the pH of the clear supernatant solution was adjusted to 7.5 with 2N ammonia, and 5 g ammonium sulfate was added per 10 ml. The precipitate was collected by centrifugation after 15 min and washed three times with the original volume of a 50% ammonium sulfate solution in 0.4 M Tris-HCl, pH 7.8. The precipitate was then dissolved in 0.1 M Tris-HCl, pH 7.8, corresponding to the original volume. The solution contained about 0.8 mg protein per ml. It was stored frozen at -15°.

Two types of assay were used to measure the reaction at 37°. One could either follow the formation of ^{14}C-labeled product or of orthophosphate. In the former assay one volume of enzyme (25 or 50 µl) was added to a mixture at 37° of one volume water and two volumes of a solution containing 0.2 µc of uniformly labeled ^{14}C-serine, 120 mc/mM (New England Nuclear), together with 0.02 M L-serine, 0.02 M sodium pyrophosphate, 0.004 M $MgSO_4$, and 0.4 M Tris-HCl, pH 7.8. After varying times the solution was placed in boiling water. Ionophoresis of 25 µl aliquots was performed at pH 8.6, using Whatman No. 1 paper strips and veronal sodium hydroxide buffer (0.038 µ) in the Model 50 apparatus of the Kensington Scientific Corporation. The solution was applied 3 cm from the cathode ends of the paper strips. After application of a voltage gradient of 17.5 volts per cm for one hour the serine had migrated about 3 cm and a new radioactive region about 8 cm towards the anode. Radioactivity was determined on the dried paper in a Nuclear Chicago Actigraph III strip counter equipped with a Model 8735 digital integrator (see Friedmann,

1968). The extent of the reaction was measured in terms of the ratio of counts of product to total counts.

When, alternatively, orthophosphate was measured (Hurst, 1964), the same conditions of incubation were employed, except that the radioactive serine was usually omitted.

The stoichiometry of the reaction is summarized in Table I. There

TABLE I

COMPARISON OF PHOSPHOSERINE AND ORTHOPHOSPHATE FORMATION

Incubation Time min	Radioactivity				Phosphate		
	serine[1]	P-serine[1]	total	$\frac{\text{P-serine}}{\text{total}}$	A[2]	μmoles P_i per ml reaction mixture	$\frac{2[P_i]^3}{\text{total } [P_i]}$
10	9925	1898	11823	0.16	0.042	1.54	0.15
20	8062	3450	11512	0.30	0.079	2.91	0.29
30	6880	4967	11847	0.42	0.115	4.23	0.42
40	5101	6657	11758	0.57	0.158	5.82	0.58

[1]The numbers are the counts in 25 μl reaction mixture registered over the respective radioactive region, corrected for background. The paper strips moved at 30 cm/hr past a slit 6 mm wide. The reaction mixture was as indicated in the text, except that sodium fluoride was used instead of water to give a final concentration of 0.01 M.

[2]Absorbance due to liberated phosphate was measured according to Hurst (1964) at 700 mμ against a reaction blank which lacked serine and which was inactivated by boiling immediately after enzyme addition. The color from 10 μl aliquots was read after addition of reagents in a final volume of 10 ml.

[3]Total phosphate was determined by incubating the reaction mixture containing $MgSO_4$ at a final concentration of 0.01 M, and 5 μl yeast inorganic pyrophosphatase (Worthington) instead of *P. shermanii* enzyme, for 3 hr at room temperature.

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is strict correspondence between the amount of total radioactivity converted to product and the proportion of pyrophosphate converted to free orthophosphate. All radioactivity was accounted for as the sum of counts in serine and in product. The reaction was 97% complete in 90 min at 37°. No product was formed in the absence of pyrophosphate or of enzyme. The amount of product formed in 10 min was proportional to the amount of enzyme added. The product is tentatively identified as O-phospho-L-serine

on the basis of the following: after ionophoresis on paper at pH 8.6 (above) or at pH 2.8 (0.5 N acetic acid) the position of the radioactive, ninhydrin-positive product coincided with that of authentic O-phospho-L-serine (Sigma). On incubation at 30° with *E. coli* alkaline phosphatase the product disappeared completely, and the corresponding amount of radioactivity re-appeared in the serine region. The amount of radioactivity in the product was inversely proportional to the concentration of L-serine added and was not affected by the presence of equimolar amounts of threonine, ethanolamine or oxalacetate. Potential phosphate donors such as ATP, ADP, GTP, UTP, CTP were inactive.

It is tentatively concluded that the reaction product is O-phospho-L-serine and not a decomposition product of serine, and that pyrophosphate is the specific phosphate donor. Experiments are in progress to confirm the identity of the product by kinetic and isolation experiments.

The enzyme solution possesses some additional activities which may or may not be associated with the phosphotransferase. These are: (1) a pyrophosphatase which is strongly magnesium dependent. Little pyrophosphate is hydrolyzed in the absence of serine under standard conditions, but hydrolysis of pyrophosphate becomes quite rapid if the final concentration of magnesium ion is raised to 0.01 M. (2) Phosphoserine:serine phosphotransferase. The radioactivity of a small amount of ^{14}C-serine is rapidly equalized between serine and phosphoserine when added to a solution containing 0.01 M of each of these compounds, 0.01 M magnesium ion and enzyme. Smaller concentrations of magnesium will also support activity, but at a slower rate. (3) Phosphoserine phosphatase, variable in different preparations. At a concentration of 0.01 M, sodium fluoride inhibits these three activities, but not the pyrophosphate-serine phosphotransferase.

Inorganic pyrophosphate is formed in many enzymatic reactions (see review by Kornberg, 1957). The present reaction differs in major respects from those utilizing pyrophosphate which have been described before. Thus

for the phosphorylation of glucose by liver microsomal preparations (Rafter, 1960; Stetten and Rounbehler, 1968) both ionorganic pyrophosphate and ATP are active, while serine is not a substrate. The present enzyme is inactive with glucose. In the phosphorylation of oxalacetate (Lochmüller *et al.*, 1966; Wood *et al.*, 1966) the substrate is not a primary alcohol. It is of interest that homoserine kinase requires ATP and does not act on serine (Watanabe *et al.*, 1957). The possible functions and distribution of the enzyme are under investigation. It is possible that this enzyme serves to mobilize serine for gluconeogenesis. It might perhaps also play a role in phospholipid biosynthesis. It is tempting to speculate that as in the case of the phosphoenolpyruvate carboxytransphosphorylase, this enzyme helps to remove pyrophosphate generated biosynthetically, thus accounting in part for the high yields of this microorganism (Bauchop and Elsden, 1960; Wood *et al.*, 1966). Appreciable amounts of serine are present in the medium, containing peptides and amino acids derived from casein. The possibly adaptive nature of the enzyme has not been investigated thus far.

ACKNOWLEDGEMENTS

This research was supported by Research Grant No. AM-09134 from the National Institutes of Health, USPHS, the USPHS General Research Support Grant No. FR 5367, and the USPHS Training Grant No. 5T1 GM-424.

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