BBA 66100

FURTHER STUDIES ON THE MECHANISM OF ACTION OF AN ACID PHOSPHATASE FROM BAKER'S YEAST (*SACCHAROMYCES CEREVISIAE*)

INFORMATION FROM EXPERIMENTS ON TRANSPHOSPHORYLATION

P. BOER AND ELIZABETH P. STEYN-PARVÉ
Laboratory for Physiological Chemistry, The University, Utrecht (The Netherlands)

(Received November 3rd, 1969)

SUMMARY

1. Two possible working hypotheses are considered for the mechanism of action of the yeast acid phosphatase: a covalent double displacement mechanism as presumed to apply to *e.g.* the alkaline phosphatases, and a mechanism analogous to the non-enzymatic hydrolysis of phosphomonoesters. To distinguish between these two we have looked for the occurrence of a phosphoryl enzyme as an intermediate in the reaction and have studied the transphosphorylation characteristics of the enzyme.

2. We were not able to isolate a radioactive enzyme after incubation with radioactive inorganic phosphate.

3. The transphosphorylation behaviour of the yeast acid phosphatase, expressed in a new manner, closely resembles that of the non-enzymatic process, and is quite different from that of several other acid and alkaline phosphatases.

4. We conclude that a single displacement mechanism analogous to the non-enzymatic hydrolysis of the phosphomonoester monoanion is preferable as working hypothesis for the action of the yeast acid phosphatase. At present this is the only phosphatase for which such a mechanism can be advanced.

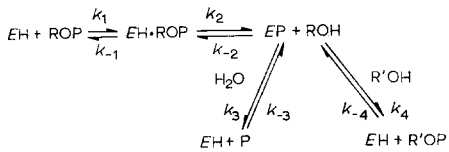
INTRODUCTION

As part of a comparative study of the similarities and differences between the two nonspecific phosphomonoesterases (an acid phosphatase (EC 3.1.3.2), pH optimum 3–4, and an alkaline one (EC 3.1.3.1), pH optimum 8–9) of baker's yeast on a molecular level[1,2], we are investigating the mechanism of action of the acid phosphatase.

In contrast to the alkaline phosphomonoesterases[3–5], little is known about the mechanism of action of acid phosphomonoesterases in general. ALBERS *et al.*[6] have

suggested a mechanism for the yeast acid phosphatase, but we were able to show that it is probably not correct[7].

Two possibilities present themselves. The first would be analogous to the covalent double displacement mechanism[8] presumed to apply to several hydrolytic enzymes[9], including the alkaline phosphatases, the so-called serine esterases. This mechanism can be represented by the following scheme:



The enzyme EH forms an enzyme–substrate complex with the phosphomonoester ROP, which first dissociates into a phosphoryl enzyme EP and an alcohol ROH. The phosphoryl enzyme, in which the phosphoryl group is covalently bound to an amino acid residue (serine) in the enzyme protein, can next either be dissociated by water, giving the free enzyme EH and inorganic phosphate, P_i, or by transferring the phosphoryl group to a suitable acceptor alcohol R'OH, in which event free enzyme and a phosphomonoester R'OP are formed.

The second possibility is a mechanism analogous to the non-enzymatic hydrolysis of phosphomonoesters. The latter process has been extensively examined by several investigators[10,11], who have proposed a mechanism that can be summarized as follows:

$$ROPO_3H^- \text{ - - - } \rightarrow ROH + [PO_3]^-$$

$$[PO_3]^- + H_2O \rightarrow H_2PO_4^-$$

In this scheme the phosphomonoester monoanion ($ROPO_3H^-$), the only species for which this mechanism holds, dissociates *via* various intermediates into an alcohol ROH and an unstable monomer metaphosphate ion $[PO_3]^-$, the latter then reacting rapidly with water. The pH optimum for the reaction is about 4, which may be ascribed to the relative instability of the monoanion.

In the pH-stability range (3.5–5.5) of the yeast acid phosphatase phosphomonoesters are mainly present as monoanions[12]. For the enzymatic reaction therefore a non-covalent single displacement mechanism[8] along these lines is also conceivable, in which the effect of the enzyme is to lower the activation energy.

There are several means for distinguishing between these two possible mechanisms.

In the first place, one can look for the occurrence of a phosphoryl enzyme as intermediate in the reaction. If this is found, it argues in favour of the first, covalent mechanism[3,13].

Secondly, one can examine the transphosphorylation process. Several phosphomonoesterases, both acid[14–16] and alkaline[17], possess this property. Moreover, CHANLEY AND FEAGUSON[18] have shown that, during non-enzymatic hydrolysis of the phosphomonoester monoanion in mixtures of methanol and water, some transphosphorylation also takes place, but that the characteristics of this process are quite different from those observed for several acid and alkaline phosphomonoesterases.

It should therefore be possible to decide whether the reaction catalysed by the yeast acid phosphatase follows the pattern of other enzymatic transphosphorylations or that of the non-enzymatic process.

We first looked for an intermediate phosphoryl enzyme[19] by trying to label the yeast acid phosphatase with radioactive inorganic phosphate ($^{32}P_i$) at pH 5.0 according to ENGSTRÖM[13]. However, we were unable to isolate a radioactive enzyme, either when working under acid conditions after the incubation, or under alkaline conditions. This is probably not due to faulty experimentation, because in similar experiments with the alkaline phosphatase of *Escherichia coli* we were able to confirm the results of others[3,13] and to find labelled phosphoserine after acid hydrolysis of the radioactive enzyme protein.

We then turned to an investigation of transphosphorylation. From the results obtained, which are reported below, a non-covalent single displacement mechanism seems most likely.

MATERIALS AND METHODS

For our experiments we used dilutions of a single preparation of yeast acid phosphatase, purified as described by BOER AND STEYN-PARVÉ[2]. Both substrate *p*-nitrophenyl phosphate and enzyme were dissolved in 0.1 M acetate buffer. When necessary, the pH was adjusted to the required value. In this report one enzyme unit is defined as the amount which will hydrolyse 1 μmole *p*-nitrophenyl phosphate per min in 0.1 M acetate buffer (pH 3.8) at 30°.

Transphosphorylation experiments were performed as follows. The incubation mixture (0.5 ml containing 11 μmoles *p*-nitrophenyl phosphate per ml, an appropriate amount of an acceptor and 0.17 enzyme units/ml) was incubated in a glass tube, closed with a rubber stopper, at 30° during a fixed time. The reaction was then stopped by adding 1.5 ml 0.5 M H_2SO_4. Samples from this solution were mixed with a known amount (excess) of 2 M NaOH and the absorbancy of *p*-nitrophenol was measured at 401 nm. The amount of *p*-nitrophenol liberated in the incubation mixture was calculated from known dilutions and the molar extinction coefficient of this compound. The amount of P_i liberated was estimated in other samples of the same acidified incubation mixture, according to the method of TAUSSKY AND SHORR[20].

To measure the velocity of hydrolysis we also incubated reaction mixtures from which the acceptor was omitted, while the usual blank runs (incubation mixtures without enzyme or substrate) were performed.

The comparison between enzymatic and non-enzymatic transphosphorylation was performed as follows.

For enzymatic transphosphorylation the procedure described above was followed, except that 0.29 enzyme unit/ml was used, and 0, 4.8 or 10.1 mole % methanol were added as acceptor. The incubation time was 2 h, and the pH of the buffer solution was 3.8. We have ascertained that methanol, in these concentrations, does not interfere with the estimation of P_i according to the method of TAUSSKY AND SHORR[20].

For the non-enzymatic transphosphorylation, incubation mixtures with the same concentrations of *p*-nitrophenyl phosphate and methanol were used, but the total volume was 5 ml. Immediately after the components were mixed, samples of

0.5 ml were taken in which the amounts of *p*-nitrophenol and P_i already present in the incubation mixture could be estimated. After the mixture had been incubated for 120 h, samples were again taken in which the reaction was stopped by adding 0.5 M H_2SO_4, and the amounts of *p*-nitrophenol and P_i were estimated. Thus we could calculate the amounts of *p*-nitrophenol and P_i liberated during the incubation. The incubation experiments were performed in duplicate, as were the analyses.

The degree of transphosphorylation we express as follows:

$$\% \text{ Transphosphorylation} = \frac{[p\text{-nitrophenol}]_r - [P_i]_r}{[p\text{-nitrophenol}]_r} \times 100$$

The concentrations of *p*-nitrophenol and inorganic P_i liberated are expressed as relative concentrations, $[p\text{-nitrophenol}]_r$ and $[P_i]_r$. In the absence of an acceptor the concentrations of *p*-nitrophenol and P_i liberated are by definition equal and have the value 100. In presence of an acceptor the concentrations of *p*-nitrophenol and P_i liberated during incubation under the same conditions, are based on the concentration of the corresponding product obtained without acceptor.

The advantage of this manner of expression is that it makes comparison of results easier; furthermore, it avoids rather large deviations in the percentage of transphosphorylation caused by relatively small differences between the results of *p*-nitrophenol and P_i analyses, especially when there is not much difference between the concentrations of *p*-nitrophenol and P_i liberated in presence of an acceptor. The absolute values of the concentrations of the products liberated are also stated.

RESULTS

Transphosphorylation by the yeast acid phosphatase was first examined under various conditions, using different amounts of glucose, Tris and methanol as acceptors, and varying the reaction times. Experiments were performed at two different pH's (3.8 and 5.2), as the degree of transphosphorylation is often dependent on pH (refs. 15–17). The results are presented in Table I.

It can be seen that in all circumstances the addition of an acceptor did not increase the rate of reaction; with Tris and methanol it was even markedly lower. In all experiments there was only very little transphosphorylation, of the order of the experimental error.

Table II shows the results of an experiment in which enzymatic and non-enzymatic transphosphorylation were compared with methanol as acceptor. In the presence of the enzyme the amount of products formed in 2 h was about four times that formed without enzyme in 120 h. Addition of methanol lowered the rate of liberation of products, and caused a difference between the amounts of *p*-nitrophenol and P_i liberated.

For more convenient comparison the values in Table II have been converted to relative concentrations. These are summarized in Table III. The percentages of transphosphorylation are also given. It is obvious that the trend in the relative concentrations of *p*-nitrophenol and P_i liberated as a function of the amount of methanol added was the same for both the enzymatic and the non-enzymatic process. Consequently, we found for each concentration of methanol closely similar percentages of transphosphorylation for both processes.

TABLE I

SUMMARY OF EXPERIMENTS ON TRANSPHOSPHORYLATION BY ACID PHOSPHATASE FROM BAKER'S YEAST

Acceptor	*Acceptor concn. (M)*	*pH*	*Incubation time*	*Presence (+) or absence (−) of acceptor*	*Concn. (μmoles/ml) of liberated product*		*Rel. concn. liberated product*		*Transphosphorylation (%)*
					p-Nitrophenol	*P_i*	*p-Nitrophenol*	*P_i*	
Glucose	0.8	3.8	10 min	−	1.7	1.7	100	100	
				+	1.7	1.6	100	94	6
		3.8	1 h	−	4.8	4.8	100	100	
				+	4.4	4.3	92	90	2
		3.8	4 h	−	9.0	8.0	100	100	
				+	8.3	7.4	92	92	0
		3.8	5.25 h	−	8.7	8.9	100	100	
				+	8.4	8.2	98	92	6
	0.22	5.2	0.5 h	−	2.6	2.6	100	100	
				+	2.6	2.5	100	98	2
		5.2	2 h	−	6.0	6.0	100	100	
				+	6.0	5.9	100	98	2
Tris	0.8	5.2	37 min	−	3.3	3.2	100	100	
				+	2.1	2.1	64	67	0
Methanol	2.5	5.2	2.25 h	−	7.0	7.0	100	100	
				+	6.2	5.7	89	84	6

DISCUSSION

The experiments described above show that the transphosphorylation characteristics of the yeast acid phosphatase closely resemble those of the non-enzymatic process[10,11]. CHANLEY AND FEAGUSON[18], who studied non-enzymatic transphosphory-

TABLE II

COMPARISON BETWEEN NON-ENZYMATIC TRANSPHOSPHORYLATION AND TRANSPHOSPHORYLATION CATALYZED BY THE ACID PHOSPHATASE FROM BAKER'S YEAST

Averages and standard deviations calculated from duplicate incubations, followed by duplicate determinations of liberated products. Absolute values. (See also Table III.) Donor, *p*-nitrophenol phosphate; acceptor, methanol.

Methanol (mole %)	*Concn. (μmoles/ml) of liberated products*			
	Non-enzymatic transphosphorylation (120 h)		*Enzymatic transphosphorylation (2 h)*	
	p-Nitrophenol	*P_i*	*p-Nitrophenol*	*P_i*
0	0.90 ± 0.01	0.87 ± 0.02	3.46 ± 0.07	3.34 ± 0.07
4.8	0.80 ± 0.01	0.71 ± 0.02	3.26 ± 0.07	2.84 ± 0.06
10.1	0.69 ± 0.01	0.56 ± 0.01	2.21 ± 0.05	2.14 ± 0.04

TABLE III

COMPARISON BETWEEN NON-ENZYMATIC TRANSPHOSPHORYLATION AND TRANSPHOSPHORYLATION CATALYZED BY THE ACID PHOSPHATASE FROM BAKER'S YEAST

Relative values (averages with standard deviations). (See also Table II) Donor. *p*-nitrophenyl phosphate; acceptor, methanol.

Methanol (mole %)	*Rel. concn. of liberated p-nitrophenol*		*Rel. concn. of liberated P_i*		*Transphosphorylation (%)*	
	Non-enzymatic (120 h)	*Enzymatic (2 h)*	*Non-enzymatic (120 h)*	*Enzymatic (2 h)*	*Non-enzymatic (120 h)*	*Enzymatic (2h)*
0	100	100	100	100		
4.8	89 ± 1	94 ± 2	81 ± 2	85 ± 2	9 ± 2	9 ± 2
10.1	77 ± 1	82 ± 2	64 ± 1	64 ± 1	17 ± 2	21 ± 3

lation during hydrolysis of the phosphomonoester monoanion in mixtures of alcohols and water, observed that the velocity of hydrolysis of *p*-nitrophenyl phosphate and phenyl phosphate decreases with increasing mole percentage of methanol and ethanol, and that the percentage of transphosphorylation is of the order of the mole percentage of methanol in the mixture.

Our results confirm these observations for the non-enzymatic process and show that the yeast acid phosphatase behaves in the same manner, though of course in the presence of enzyme the reaction proceeds at a much higher rate. This manner is quite different from that of the transphosphorylation by the alkaline phosphatase from *E.*

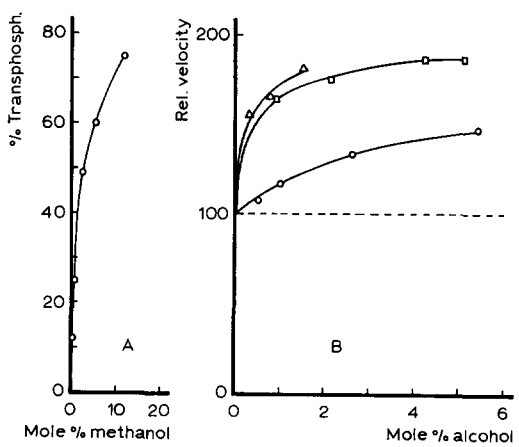


Fig. 1. A. Percentage of transphosphorylation by acid phosphatase from citrus fruit as a function of the mole percentage of methanol in the incubation mixture. Substrate, *p*-nitrophenyl phosphate. (Adaptation of AXELROD's[15] data.) B, Relative velocity of hydrolysis of the substrate as a function of the mole percentage of alcohol in the incubation mixture for the acid phosphatase from citrus fruit and the acid prostate phosphatase. △—△, acid phosphatase from citrus fruit; substrate, phenolphthalein phosphate; acceptor, ethanol (data, AXELROD[15]); ○—○, acid phosphatase from citrus fruit; substrate, *p*-nitrophenyl phosphate; acceptor, methanol (data, AXELROD[15]); □—□, acid phosphatase from prostate; substrate phenolphthalein phosphate; acceptor, ethanol (data APPLEYARD[14]).

coli[17] and several acid phosphatases (from citrus fruit[15], human prostate[14] and erythrocytes[21]). For these enzymes a much higher percentage of transphosphorylation is found (up to 60–70%), exceeding by far the order of the mole percentage of acceptor alcohol present, while the rate of decomposition of the substrate increases with increasing concentrations of acceptor. This is clearly inferred from Fig. 1, in which we have graphically summarized some data from the literature[14,15]. These considerations, together with our inability to show the occurrence of a phosphoryl enzyme intermediate, lead us to prefer a non-covalent single displacement mechanism to a covalent double displacement mechanism as a working hypothesis for the action of the yeast acid phosphatase.

Such a mechanism could be schematically outlined as follows:

$$E + ROPO_3H^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot ROPO_3H^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + ROH + [PO_3]^-$$

$$[PO_3]^- + H_2O \xrightarrow{k_3} H_2PO_4^-$$

$$[PO_3]^- + CH_3OH \xrightarrow{k_4} CH_3OPO_3H^-$$

The products of the enzyme-catalysed reaction are an alcohol and the unstable monomer metaphosphate ion $[PO_3]^-$. The latter reacts rapidly and spontaneously with water or (*e.g.*) methanol.

BUNTON *et al.*[22] proposed that the acid phosphatase from human prostate would act according to a mechanism analogous to the non-enzymatic hydrolysis of the phosphomonoester monoanion. We believe this is most unlikely.

Firstly, as already mentioned, the transphosphorylation behaviour of this enzyme is quite different from the non-enzymatic process; in this respect it bears more resemblance to the alkaline serine phosphatases which are presumed to act by a covalent double displacement mechanism. Secondly, GREENBERG AND NACHMANSOHN[23] have obtained a marked incorporation of radiocative P_i into this phosphatase, although they were not able to isolate labelled phosphoserine, and therefore presumed the phosphoryl group would be attached to some other amino acid residue. This argues in favour of the occurrence of a phosphoryl enzyme intermediate, and therefore in this respect, too, there is more resemblance to the alkaline phosphatases.

Therefore our conclusion is that at present the only enzyme for which a mechanism analogous to the non-enzymatic hydrolysis of the phosphomonoester monoanion can with reason be advanced is the yeast acid phosphatase.

REFERENCES

1 G. J. M. TONINO AND E. P. STEYN-PARVÉ, *Biochim. Biophys. Acta*, 67 (1963) 453.
2 P. BOER AND E. P. STEYN-PARVÉ, *Biochim. Biophys. Acta*, 128 (1966) 400.
3 J. H. SCHWARTZ, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 871.
4 H. N. FERNLEY AND P. G. WALKER, *Biochem. J.*, 111 (1969) 187.
5 T. E. BARMAN AND H. GUTFREUND, *Biochem. J.*, 101 (1966) 460.
6 H. ALBERS, G. BUSING AND G. SCHUDT, *Enzymologia*, 30 (1966) 149.
7 P. BOER AND E. P. STEYN-PARVÉ, *Biochim. Biophys. Acta*, 171 (1969) 360.
8 D. E. KOSHLAND JR., in B. GLASS AND W. D. MCELROY, *Mechanisms of Enzyme Action*, Johns Hopkins, Baltimore, 1954, p. 608.
9 H. GUTFREUND, *An Introduction to the Study of Enzymes*, Blackwell, Oxford, 1965, p. 58.
10 TH. C. BRUICE AND S. BENKOVIC, *Bioorganic Mechanisms*, Vol. 2, Benjamin, New York, 1966, p. 1.

11 W. P. JENCKS, *Brookhaven Symp. Biol.*, 15 (1962) 143.
12 J. R. VAN WAZER, *Phosphorus and its Compounds*, Vol. 1, Wiley, New York, 1958, p. 581.
13 L. ENGSTRÖM, *Biochim. Biophys. Acta*, 56 (1962) 606.
14 J. APPLEYARD, *Biochem. J.*, 42 (1948) 596.
15 B. AXELROD, *J. Biol. Chem.*, 172 (1948) 1.
16 R. K. MORTON, *Biochem. J.*, 70 (1958) 150.
17 I. B. WILSON, J. DAYAN AND K. CYR, *J. Biol. Chem.*, 239 (1964) 4182.
18 J. D. CHANLEY AND E. FEAGUSON, *J. Am. Chem. Soc.*, 85 (1963) 1181.
19 P. BOER, Thesis, Utrecht, 1969.
20 H. H. TAUSSKY AND E. SHORR, *J. Biol. Chem.*, 202 (1953) 675.
21 M. IIO, T. HASHIMOTO AND H. YOSHIKAWA, *J. Biochem.*, 55 (1964) 321.
22 C. A. BUNTON, B. L. SILVER AND C. A. VERNON, *Proc. Chem. Soc.*, (1957) 348.
23 H. GREENBERG AND D. NACHMANSOHN, *J. Biol. Chem.*, 240 (1965) 1639.