APPARENT CHANGE IN REACTION MECHANISM OF PHOSPHOGLYCERATE MUTASE INDUCED BY SALT

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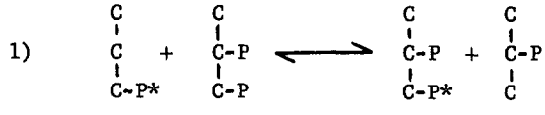
The biological role of 2,3 P-glycerate had been open to speculation until it was shown by Sutherland et al. (1949) that it behaved as an intermediate in the enzymatic interconversion of 3 P-glycerate and 2 P-glycerate. The stimulation of phosphoglycerate mutase by 2,3 phosphoglycerate was interpreted, in analogy to phosphoglucomutase, Leloir et al. (1948), to indicate an obligatory phosphate donor role for 2,3 P-glycerate with 3 and 2 phosphoglycerate as per reaction 1):

1) 

Reaction 1 implies that: a) (^{32}P) 3 P-glycerate should be converted into 2,3 P-glycerate. b) 2,3 P-glycerate should become radioactive during the course of the reaction so that its specific activity should increase in a predictable fashion. By appropriate tracer experiments the mechanism can be checked.

The discovery of P-glycerate mutases which do not require 2,3 P-glycerate, Grisolia (1964), cast some doubts on the necessary role of 2,3 P-glycerate for mutase action. This communication demonstrates that 2,3 P-glycerate does not participate as an obligatory intermediate, as shown in reaction 1, for P-glycerate mutase action. Further, it demonstrates that moderate salt concentrations affect the reaction so remarkably that two different mechanisms can be proposed. The present findings are of interest, then, not only for P-glyceromutase but because they may apply also to other hitherto unsuspected enzymatic reactions.

Radioactive 3 P-glycerate was prepared as previously described, Grisolia et al. (1961). Pyruvate was determined by the procedure of Friedemann and Haugen, (1943). P-glycerate mutase from yeast was prepared by the method of Rodwell et al. (1956) and from chicken breast muscle by that of Torralba and Grisolia (1966).

All experiments were carried out at 30°. The components, per milliter of incubation mixture, were as follows: 1.5 μmoles of (^{32}P) 3 P-glycerate, 3.3 units of enolase, Towne et al. (1957), 3.5 μmoles of ADP, 5 μmoles of $MgSO_4$, 10 μmoles of KCl, 35 μmoles Tris-Cl pH 7.4, 6.6 μmoles 2,3 P-glycerate, 8 μgrams of pyruvic kinase and 0.1 mg serum albumin. Mutase was added at 0 time in the amounts indicated in the Tables. At 0 time and at the indicated times 1.0 ml portions were withdrawn, 0.5 ml of 1 M $HClO_4$ added, centrifuged and portions of the supernatant fluids assayed for pyruvate. In theory 1.5 μmoles pyruvate per ml of the original incubation may be found at the completion of the reaction. (Under these experimental conditions there was an excess of pyruvic kinase and of enolase and all other necessary reagents so that the 2 P-glycerate formed was immediately converted to P-enol-pyruvate and its phosphate transferred to ATP; as indicated below, this was then hydrolyzed to Pi). Another portion was hydrolyzed by heating for 7 min at 100°. The samples were neutralized with KOH, centrifuged and then two 50 μl aliquots from each sample (to facilitate identification 0.1, 0.2 and 0.3 μmoles of non radioactive 2,3 P-glycerate, 3 P-glycerate and Pi respectively were added) chromatographed on Whatman # 1 paper (prewashed successively with KCl and EDTA). The chromatogram was developed overnight at room temperature with ethyl acetate-acetic acid-water (3:3:1). Detection was carried out with 1 % $FeCl_3$ in 75% ethanol followed by 1% potassium sulfosalycylate. The phosphate compounds appeared as white spots.

Spots corresponding to 2,3 P-glycerate, 3 P-glycerate, and Pi were cut and set inside the counting vial. About 1 ml of the scintillation solution (4 g of 2,5-bis-2-(5-tert-Butylbenzoxazolyl)-Thiophene per liter of toluene) was added and allowed to soak through the paper. More scintillation solution was then added to fully cover the paper (usually 17 ml were enough). The vials were then shaken gently but thoroughly and counted. The remainder of the paper chromatogram was checked and found to contain no radioactivity above the background. There was excellent agreement between the duplicates. All samples have been corrected for small i.e. 2%, hydrolysis at 0 time.

Table I demonstrates both with yeast and muscle preparations that 2 P-glycerate is labeled progressively (during 40 minutes) from (^{32}P) 3 P-glycerate 4 to 6 times faster than 2,3 P-glycerate; theoretically, according to reaction 1 and under the conditions used this should not be possible.

Table I. THE EFFECT OF TIME ON DISTRIBUTION OF (^{32}P) 3 P-GLYCERATE INTO 2,3 P-GLYCERATE AND 2 P-GLYCERATE WITH YEAST AND MUSCLE P-GLYCERATE MUTASES

The conditions were as described in the text, except that 1.66 μmoles of (^{32}P) 3 P-glycerate and 0.08 μgs of muscle or of yeast mutase per ml of incubation were used.

Time	% ^{32}P in						Pyruvate formed	
	3 P-glycerate		2,3 P-glycerate		2 P-glycerate			
min.							μmoles	
3	92	(92)*	1	(2)	7	(6)	0.14	(0.3)
5	88	(83)	2	(3)	10	(15)	0.21	(0.4)
10	73	(72)	4	(5)	23	(23)	0.53	(0.63)
15	64		5		31		0.72	
18		(50)		(9)		(41)		(0.95)
20	54	(46)	8	(7)	38	(47)	0.96	(0.99)
30	33	(30)	10	(13)	57	(57)	1.24	(1.25)
40	23	(15)	14	(13)	63	(72)	1.58	(1.51)

* The figures in parentheses were obtained with yeast mutase.

As exemplified in Table II and at several levels of 2,3 P-glycerate, up to about twice the concentration in the erythrocyte, Towne et al. (1957), the fixation occurs preferentially on 2 P-glycerate.

Table II. EFFECT OF 2,3 P-GLYCERATE ON (^{32}P) 3 P-GLYCERATE DISTRIBUTION INTO PHOSPHOGLYCERATES

The conditions were as described in the text, except that 2,3 P-glycerate was added as indicated. 0.08 μgs of muscle mutase per ml were used. 30 minutes incubation.

2,3 P-glycerate added per ml of incubation	% ^{32}P into			Pyruvate formed
	3 P-glycerate	2,3 P-glycerate	2 P-glycerate	
μmoles				μmoles
0.8	6	7	87	1.2
2	7	7	86	1.2
4	11	8	81	1.2
6.6	22	8	70	1.1
13.2	21	12*	67	1.1

* The slight increase is probably due to higher ionic strength (see below).

Towne (1960), reported that arsenate can replace 2,3 P-glycerate as an activator with 2,3 P-glycerate dependent enzymes. We have shown that arsenate behaves as a cofactor and also as a competitive inhibitor for 2,3 P-glycerate. It was reasoned, therefore, that in the presence of arsenate, it should be possible to decrease even more the distribution of radioactive 3 P-glycerate into 2,3 P-glycerate. That is, if 2,3 P-glycerate acts simply as an activator, for example by changing the conformation of the protein, it should be possible to bypass exchange with this reagent entirely. As exemplified in Table III with both yeast and muscle mutase, increasing the ionic strength changes the pattern of labeling; arsenate (as well as the other salts) favors fixation on 2,3 P-glycerate.

Table III. EFFECT OF SALT ON DISTRIBUTION OF (^{32}P) 3 P-GLYCERATE INTO 2,3 P-GLYCERATE AND 2 P-GLYCERATE WITH YEAST AND MUSCLE MUTASES

The conditions were as described in the text. 0.04 µgs of yeast or of muscle P-glycerate mutase per ml were used. 30 min incubation. When used, potassium arsenate, phosphate or sulfate at pH 7.4 were added.

Mutase Source	Additions	% ^{32}P into			µmoles Pyruvate formed
		3 P-glycerate	2,3 P-glycerate	2 P-glycerate	
Yeast	None*	14	3	83	0.9
"	None	22	8	70	0.8
"	100 µmoles Phosphate	46	27	27	0.6
Yeast	200 µmoles Sulfate	57	30	13	0.5
"	100 µmoles Arsenate	41	41	18	0.5
"	475 µmoles KCl	39	58	3	0.5
Muscle	None	27	8	65	1.1
"	120 µmoles Sulfate	52	19	29	0.7
"	100 µmoles Phosphate	53	16	31	0.7
Muscle	100 µmoles Arsenate	50	21	29	0.6
"	475 µmoles KCl	26	71	13	0.9

* These samples were run without the standard additions of Tris-KCl (see text).

As shown above, we found exactly the opposite of the effect we anticipated. Indeed, at the high levels of KCl shown in the table, there is

negligible fixation on 2 P-glycerate. Therefore, in this latter case the distribution of labeling is now as predicted by equation 1. It should be noted that the salts used, other than arsenate, cannot replace 2,3 P-glycerate in the mutase reaction; moreover all are somewhat inhibitory.

In view of the extensive formation of pyruvate in all cases, the change in mechanism induced by salt cannot be due to abortive complex formation, nor is it likely to be a simple buttressing effect modifying the cofactor action of 2,3 P-glycerate. While the intimate mechanism remains to be elucidated, the unique effect demonstrated here indicates that at low salt concentration 2,3 P-glycerate may change P-glycerate mutase to an active conformation manifesting little or no exchange with 2,3 P-glycerate; the enzyme may now approach the mechanism of the 2,3 P-glycerate independent mutases. On the other hand, in the presence of high salt concentration, the mechanism must follow (whether or not involving P-enzyme formation) that proposed by Sutherland et al. (1949).

It has been proposed that P-glycerate mutase entails P-enzyme formation. Studies with the crystalline enzyme from yeast were negative; nevertheless, studies with muscle mutase indicated that P-enzyme formation could occur during the catalysis, Grisolia et al. (1961). However, whether or not there is intermediate P-enzyme formation would not modify the findings shown here.

The present findings may provide a tool for a better understanding of the active site of P-glycerate mutase, and perhaps of other enzymes; serve to emphasize the marked influence of enviromental factors in biology; and, as indicated previously, the great caution needed in interpreting "in vitro" experiments, made under conditions far from physiological, as a reflection of cellular events, Grisolia (1965). To the knowledge of the authors there is no record in the literature of a finding similar to that reported here.

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