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Hg^{2+}-INDUCED DISSOCIATION OF EXCHANGE REACTIONS FROM CATALYTIC ACTIVITY OF 2,3-DIPHOSPHOGLYCERATE-DEPENDENT PHOSPHOGLYCEROMUTASES

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SUMMARY

1. The rate of 3-phosphoglycerate exchange with 2,3-diphosphoglycerate catalyzed by phosphoglyceromutase (EC 2.7.5.3) increases in the order, rabbit < yeast < chicken enzymes.

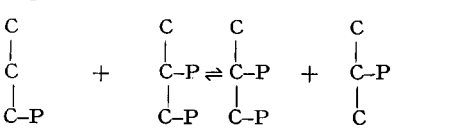
2. Treatment of the chicken and of the rabbit muscle mutase with Hg^{2+}, a procedure known to increase their 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) activity and to decrease their mutase activity, caused 149 and 294% increase, respectively, in their rate of exchange. The phosphatase activity of the yeast enzyme which is not affected by Hg^{2+} remained also unaffected after Hg^{2+} treatment in so far as exchange rates between 3-phosphoglycerate and 2,3-diphosphoglycerate.

3. The experimentally observed ^{32}P incorporation from 3-phosphoglycerate into 2,3-diphosphoglycerate was close to that theoretically expected when equilibration of the reaction was reached.

4. The phosphatase activity showed no direct relationship to the rate of the ^{32}P exchange between 3-phosphoglycerate and 2,3-diphosphoglycerate.

INTRODUCTION

Phosphoglyceromutase (2,3-diphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) catalyses the conversion of 3-phosphoglycerate (3-*P*-glycerate) to 2-phosphoglycerate (2-*P*-glycerate). This type of phosphoglyceromutase requires 2,3-P_2-glycerate and is found in animal tissues and in yeast[1]. SUTHERLAND, POSTERNAK AND CORI[2], on the basis of isotope exchange experiments, postulated an intermolecular phosphate transfer similar to that carried out by phosphoglucomutase[3], as per Reaction 1:



(1)

Abbreviation: PEP, phosphoenolpyruvate.

Radioactive 2,3-P_2-glycerate was obtained after ^{32}P-labelled 3-P-glycerate was incubated with unlabelled 2,3-P_2-glycerate in the presence of mutase. However, in none of their experiments were they able to transfer the theoretical amount of ^{32}P in 2,3-P_2-glycerate; we know now that this is a reflection of the environmental conditions, particularly salt concentration[4].

In spite of fairly extensive investigations the mechanism of action of the 2,3-P_2-glycerate-dependent mutase is not fully understood. The presence of 2,3-diphosphoglycerate phosphatase (2,3-diphospho-D-glycerate 2-phosphohydrolase, EC 3.1.3.13) activity in phosphoglyceromutase has added to the problem of elucidating the latter's mechanism of action. Recently we found that Hg^{2+} inhibit the mutase activity with concomitant activation of the phosphatase activity, and that this effect is reversible[5]. This effect was observed with the mutases from animal sources only; the mutase from yeast as well as the non-2,3-P_2-glycerate-dependent mutases remained unaffected by Hg^{2+}.

Of a fairly extensive number of kinetic and molecular parameters, only small changes in K_m and in optical rotation were found between Hg^{2+}-treated and -untreated enzyme samples. It appeared then of much interest to determine how the Hg^{2+} treatment of the mutase with the concomitant increase in phosphatase activity would affect ^{32}P exchange from 3-P-glycerate into 2,3-P_2-glycerate. The increase in phosphatase activity does change the rate of ^{32}P incorporation into 2,3-P_2-glycerate. These studies are presented in this paper.

EXPERIMENTAL PROCEDURE

Materials and methods

Phosphoglyceromutases were obtained as previously described[6].

The preparation of radioactive 2,3-P_2-glycerate was based on the work of GRISOLIA, JOYCE AND FERNANDEZ[7]. 25 ml of fresh human heparinized blood in a siliconized tube were incubated with 1 ml sodium phosphate (approx. 5 mC/ml), 0.1 ml of 0.05 M Na_3PO_4 and 0.3 ml of 1.5 M NaCl at 38° for 5 h, mixing continously by the slow bubbling of O_2–CO_2 (95:5). After centrifugation, the erythrocytes were washed twice with 15 ml of 0.15 M NaCl. 1.5 vol. of water and 2 ml of 6 M HCl were added, and then heated in a boiling-water bath for 30 min. After centrifugation the supernatant fluid was adjusted to pH 1.85 with LiOH. The solution was percolated through a column (1 cm × 9.5 cm) of Dowex 1-X 8 resin (200–400 mesh, Cl^-). The column was washed with water (approx. 50 ml) until the effluent was clear and colorless. The ^{32}P-labelled 2,3-P_2-glycerate was eluted with 10 ml of 0.5 M NaCl–0.5 M HCl, brought to pH 7 with KOH and used as such in the preparation of ^{32}P-labelled 3-P-glycerate as follows: 1.76 μmoles of ^{32}P-labelled 2,3-P_2-glycerate, 10 μmoles of 3-P-glycerate, 20 μmoles of Tris buffer (pH 7.3) and 10 units of chicken breast muscle mutase (free from enolase) in a total volume of 1.2 ml were incubated at 30° for 10 min. The mixture was heated in a boiling-water bath for 10 min, cooled and diluted with 1 ml of 0.1 M 3-P-glycerate and 2 ml of water. The radioactive 3-P-glycerate was purified using the method of BARTLETT[8]. The ^{32}P-labelled 3-P-glycerate obtained was free from 2,3-P_2-glycerate and P_i but contained 14% of ^{32}P as 2-P-glycerate (resulting from equilibration with 3-P-glycerate during incubation).

$HgCl_2$ treatment

Three mg of enzyme, 0.2 μmole of $HgCl_2$, and 50 μmoles of Tris buffer (pH 7.3) in a total volume of 0.6 ml were incubated for 5 min at 38°. The supernatant fluid, after centrifugation, was used for the exchange experiments (increasing the amount of $HgCl_2$ did not produce marked changes in phosphoglyceromutase and/or phosphatase, in confirmation of previous findings)[5].

Exchange experiments

Ten μmoles of ^{32}P-labelled 3-*P*-glycerate, 1 μmole of 2,3-P_2-glycerate, 5 μmoles of $MgSO_4$, 50 μmoles of Tris buffer (pH 7.3) and 7 units of enolase (mutase free) were mixed in a total volume of 1.6 ml. The mixture was allowed to stand at 30° until the initial 2-*P*-glycerate present in the mixture equilibrated with phosphoenolpyruvate (PEP) as observed by no further change in $A_{240\ m\mu}$. 0.1 ml of the incubation mixture was then withdrawn and 10 μl containing 2 units of mutase were added. It should be kept in mind that in order to add the same amount of mutase activity, the protein in the samples of the partially inactivated Hg^{2+}-treated animal mutases was approx. 50–100 larger than for the control samples. The reaction was followed at 30° by reading $A_{240\ m\mu}$. From time to time 0.1-ml aliquots were taken and immediately added to 20 μl of 5 M $HClO_4$. After about 2 min at room temperature they were heated in a boiling-water bath for 5 min. The samples were brought to pH 9–10 with dilute KOH using phenolphthalein as indicator (end point, pale pink color). The final volume of the samples which were now ready for chromatography was 0.2–0.3 ml.

For chromatography, prewashed Whatman No. 1 paper and *tert.*-amyl alcohol–formic acid–water (3:2:1, by vol.) were used[9]. 50 μl of the samples were chromatographed along with 0.1 μmole each of non-radioactive 2,3-P_2-glycerate and 3-*P*-glycerate (both at pH 10) for 14 h at room temperature. The phosphate compounds were detected by spraying the dried chromatogram with 0.1% $FeCl_3$ in 75% ethanol, followed by 1% sodium–potassium salt of sulfosalycylic acid[9].

Spots corresponding to 2,3-P_2-glycerate (1.5 inch × 1 inch) and mixtures of 3-*P*-glycerate, PEP and P_i (1.5 inch × 2.5 inch) were cut and the ^{32}P measured as previously described[9].

The amount of 2-*P*-glycerate formed was calculated from the measured value for PEP. Under our experimental conditions, 1.4 μmoles of 2-*P*-glycerate formed corresponded to 1 μmole of PEP measured. The molar extinction coefficient of PEP at 240 mμ was taken as $1.75 \cdot 10^3$.

Phosphatase measurements

2,3-Diphosphoglycerate phosphatase was measured by modification of a previously published procedure[10] as follows: 3 μmoles of 2,3-P_2-glycerate, 50 μmoles of Tris buffer (pH 7.4) and about 0.3 mg of the enzyme in a total volume of 1.0 ml were mixed and incubated at 38° for 30 min. The incubation was stopped by the addition of 1 ml 10% $HClO_4$, centrifuged and the supernatant fluid was assayed for P_i.

RESULTS AND DISCUSSION

Fig. 1 shows, in confirmation of previous findings[4,9], that the synthesis of 2-*P*-glycerate is faster than the ^{32}P incorporation of *P*-glycerate into 2,3-P_2-glycerate with

both non-treated and Hg^{2+}- treated rabbit muscle mutase. Moreover, as illustrated, the incorporation of ^{32}P into 2,3-P_2-glycerate with the Hg^{2+}-treated enzyme is faster than with the untreated enzyme. However, as expected, as the reaction approaches equilibrium the values obtained with the untreated enzyme approach those obtained with the Hg^{2+}-treated enzyme.

Although not illustrated for compactness of presentation, similar experiments carried out with chicken breast muscle mutase showed a greater rate of exchange of ^{32}P-labelled 3-P-glycerate and 2,3-P_2-glycerate with the Hg^{2+}-treated enzyme than

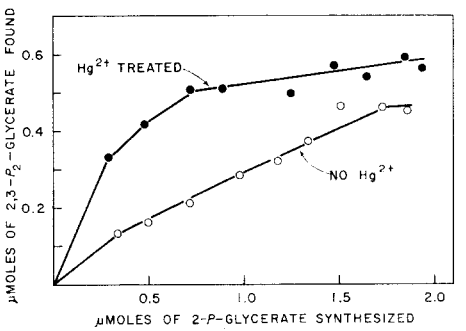


Fig. 1. The effect of Hg^{2+} on the mutase and exchange reaction of rabbit muscle phosphoglyceromutase. The conditions were as described in the text. The amounts of 2,3-P_2-glycerate found to be exchanged with 3-P-glycerate at the indicated amounts of 2-P-glycerate formed are recorded in the ordinate.

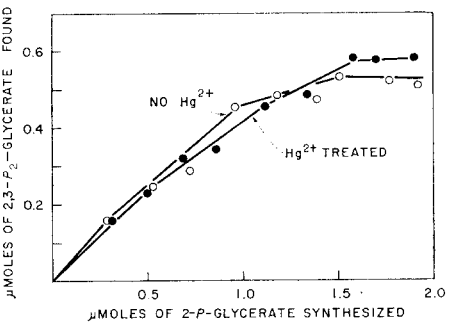
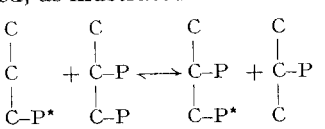


Fig. 2. The effect of Hg^{2+} on the mutase and exchange reaction of yeast phosphoglyceromutase. The conditions were as described in the text. The amounts of 2,3-P_2-glycerate found to be exchanged with 3-P-glycerate at the indicated amounts of 2-P-glycerate formed are recorded in the ordinate.

with the native mutase. Again, as expected, the amount of ^{32}P incorporated into 2,3-P_2-glycerate as the reaction approaches equilibrium, is essentially the same in both cases. It should be noted also that the rate of exchange both for the non-treated and for the Hg^{2+}-treated chicken muscle enzyme is slower than for the formation of 2-P-glycerate.

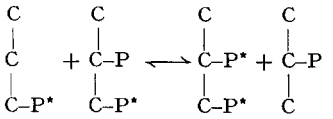
The yeast enzyme showed a different behavior. Although again the exchange rate is slower than for the synthesis of 2-P-glycerate this enzyme, which is not affected by Hg^{2+}, shows no change in the rate of exchange whether Hg^{2+} is present or not (see Fig. 2). These experiments further confirm that it is the enzyme–Hg^{2+} complex or the 'altered' enzyme, and not the Hg^{2+}, that causes the difference in the rate of exchange.

If it is assumed that the phosphoglyceromutase action proceeds as postulated by SUTHERLAND, POSTERNAK AND CORI[2], it could be expected that in a system containing an excess of ^{32}P-labelled 3-P-glycerate and 1 μmole of 2,3-P_2-glycerate, when 1 μmole of 2-P-glycerate is formed, 1 phosphate in the 2,3-P_2-glycerate should be labelled, as illustrated in Reaction 2.



(2)

When 2 μmoles of 2 *P*-glycerate are formed, both of the phosphate groups in the cofactor will be labelled as illustrated.



(3)

From here on, further synthesis of 2-*P*-glycerate will not increase the specific activity of the ^{32}P-labelled 2,3-P_2-glycerate.

In the systems used in this paper 1 ml of the incubation mixture contained 6.25 μmoles of ^{32}P-labelled 3-*P*-glycerate and 0.625 μmole of 2,3-P_2-glycerate. If the reaction proceeds as postulated by SUTHERLAND, POSTERNAK AND CORI[2], it is possible to calculate the values of ^{32}P at all stages of the reaction. Calculations were carried out from data similar to that illustrated in Figs. 1 and 2, and the experimental data agreed very closely with the theoretical. It is of interest that the amount of ^{32}P incorporated into 2,3-P_2-glycerate leveled off in all cases when about 1.7 μmoles of 2-*P*-glycerate were synthesized.

Table I presents a comparison of mutase, exchange and phosphatase activities of several phosphoglyceromutases. It appears that the Hg^{2+}-induced increase in the 2,3-diphosphoglycerate phosphatase activity of the animal mutases results in an increase in the extent of ^{32}P incorporation into 2,3-P_2-glycerate. However, there appears to be no direct relationship between the phosphatase activity and the rate of exchange into 2,3-P_2-glycerate with enzyme from different sources. With the untreated enzymes, the yeast mutase, with nearly 3 times more phosphatase activity than the rabbit and chicken enzymes, incorporated less ^{32}P into 2,3-P_2-glycerate than the chicken enzyme. Moreover, under the same conditions, the rabbit enzyme, having about the same phosphatase activity as the chicken enzyme, exchange, up to the point when 1.25 μmoles of 2-*P*-glycerate are formed, only half as much ^{32}P into 2,3-P_2-glycerate as the chicken enzyme.

As previously shown the Hg^{2+} treatment affects slightly the Michaelis constants of both the mutase and phosphatase activities of the animal enzymes[5]. It seems that the combined effect of the increased phosphatase and of the exchange rate shown in

TABLE I

THE INFLUENCE OF Hg^{2+} TREATMENT ON MUTASE, EXCHANGE AND 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITIES OF SEVERAL PHOSPHOGLYCEROMUTASES

The actual values for the enzyme preparations used were as follows: Mutase, 2400 units for all three preparations; the initial velocity of the exchange reaction was 0.35, 0.66, and 0.73 the mutase velocity for the rabbit, yeast, and chicken preparations, respectively. The 2,3-diphosphoglycerate phosphatase activity of the untreated preparations was 0.62, 0.87, and 2.3 μmoles of P_i liberated in 30 min per mg protein[9] for rabbit, chicken, and yeast preparations, respectively.

Enzyme preparation	*% of initial activity after Hg^{2+} treatment*		
	Mutase	*Exchange*	*Phosphatase*
Rabbit muscle	2	294	265
Chicken muscle	1	149	260
Yeast	100	100	100

this paper would be of opposite signs to the K_m change previously reported; that is to say, a lower affinity for the 2,3-P_2-glycerate should facilitate exchange as per Reaction 1. Thus, the most likely possibility is that one of the kinetic constants, is affected. The present findings, taken in conjunction with previous investigations[4,5,11], indicate that both the kinetic and the structural behavior of phosphoglyceromutase are affected by Hg^{2+} as well as by cofactor and salt.

It has been shown recently[11] that at both high and low ionic strength the initial velocity patterns are the same, and show a basic ping-pong pattern with competitive substrate inhibition by both substrates. We have outlined a mechanism which predicts the observed initial velocity patterns regardless of the size of the rate constants or the kinetic constants which are combinations of rate constants. We have shown that most likely at low ionic strength 2,3-P_2-glycerate may dissociate from the enzyme only once in every hundred thousand catalytic cycles, although its presence is still needed to prevent accumulation of inactive free enzyme, and possibly to convert it into an active form. At high ionic strength the dissociation of 2,3-P_2-glycerate would be so much faster than the maximum velocity that the enzyme 2,3-P_2-glycerate complex would completely exchange with free 2,3-P_2-glycerate and less label would find its way into 2-P-glycerate.

The proposed mechanism is a combination of Reaction 1 and Reaction 4, with the proportion of each depending on environmental conditions[4,11], and as shown here, on modifiers[12] such as Hg^{2+}.

3-P-glycerate $\rightleftarrows$ 2-P-glycerate (4)

It seems from these and from previous experiments that the combination of the Hg^{2+} probe with low and high salt concentration experiments at several levels of 2,3-P_2-glycerate may be of value in clarifying the mechanism of the reaction. It will be of particular interest to see if the exchange reaction can be increased above the catalytic reaction at high salt concentration and in the presence of Hg^{2+}.

ACKNOWLEDGEMENTS

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