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THE EFFECTS OF MERCURY AND OTHER REAGENTS ON PHOSPHOGLYCERATE MUTASE–2,3-DIPHOSPHOGLYCERATE PHOSPHATASE FROM KIDNEY, MUSCLE AND OTHER TISSUES

D. DIEDERICH, A. KHAN*, I. SANTOS** AND S. GRISOLIA

Departments of Biochemistry and Medicine, University of Kansas Medical Center, Kansas City, Kan. (U.S.A.)

SUMMARY

Phosphoglycerate mutase (EC 2.7.5.3) was purified 2000-fold from pork kidney. The molecular weight estimated by Sephadex gel filtration was approx. 65 000. This is in close agreement with other animal phosphoglyceromutases.

The previously observed Hg^{2+}-induced reversible transformation of chicken and rabbit muscle phosphoglycerate mutase (EC 2.7.5.3) into 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) occurs also with human skeletal muscle phosphoglycerate mutase but does not occur with purified pork kidney phosphoglycerate mutase or with preparations of phosphoglycerate mutase from beef liver, beef brain and human red cells.

The uniqueness of the behavior of muscle phosphoglycerate mutase from a number of species is illustrated. On the other hand, the insensitivity of phosphoglycerate mutase from other tissues to Hg^{2+} is typified by the kidney enzyme.

Pyrophosphate and phosphoglycolate stimulated the 2,3-diphosphoglycerate phosphatase activity of purified preparations of phosphoglycerate mutase from pork kidney and chicken breast. The mutase activity was unaffected by these reagents.

INTRODUCTION

All 2,3-diphosphoglycerate-dependent phosphoglycerate mutases (EC 2.7.5.3) thus far tested manifest 2,3-diphosphoglycerate phosphatase activity[1]. We have shown recently that Hg^{2+} inhibits phosphoglycerate mutase and at the same time, increases the 2,3-diphosphoglycerate phosphatase (EC 3.1.3.13) activity of heart and chicken breast phosphoglycerate mutase preparations[2]. It was of interest to check if Hg^{2+} exerted similar effects on the phosphoglycerate mutase of other tissues, particularly kidney, since such a Hg^{2+} effect upon kidney phosphoglycerate mutase could

* Present address: B. Kothakota (post), Madanapalti (Taluk), Chiltoor (Dt.) A.P., India.
** Present address: Faculdade de Medicina UFMG, C.P. 340, Belo Horizonte, Brazil, South America.

serve as an explanation for some of the toxic effects of Hg^{2+} in producing lower nephron nephrosis.

Experiments were carried out to determine if the mutase–phosphatase interconversion occurs *"in vivo"* and *"in vitro"* with kidney preparations. We found that kidney mutase was very resistant to Hg^{2+}, and moreover, there was no stimulation of the phosphatase activity. It was then of interest to check other mammalian mutases for Hg^{2+} effects on both their mutase and phosphatase activities. The results of these experiments are presented in this paper.

MATERIALS AND METHODS

Dithioerythritol was purchased from the Cyclo Chemical Corporation; 3-phosphoglycerate and 2,3-diphosphoglycerate were purchased from Boehringer Mannheim Corporation. Special enzyme grade $(NH_4)_2SO_4$ was obtained from Mann Research Laboratories. $Na_3{}^{32}PO_4$ was obtained from Mallinckrodt Nuclear. All other reagents were commercial products.

Unless otherwise specified all operations were carried out at 0–3°. $(NH_4)_2SO_4$ solutions were saturated and neutralized to pH 7.0 ± 0.1 by the addition of concentrated NH_4OH. All volumes refer to the initial volume for a particular step during purification.

For the *in vivo* experiments, rats weighing from 260 to 280 g were injected intraperitoneally under ether anesthesia with 0.1% $HgCl_2$ in 0.05 M phosphate buffer at pH 7.0. After 1–4 days the kidneys were removed, trimmed of fat and capsule, and then homogenized. In all cases, 10% water homogenates were prepared using either a Potter–Elvehjem homogenizer (for rat kidney) or a Waring blender (for dog kidney). The homogenates were centrifuged at 20 000 × *g* and the supernatants were retained for assay.

Pork heart, beef liver and beef brain were obtained fresh from a local slaughterhouse. The organs were cooled to 4° while being transported to the laboratory. Acetone powders were prepared as described for pork kidneys subsequently in this paper. Samples of human heart were obtained within an hour of demise; other human tissues (uterus, skeletal muscle and rib bone marrow) were obtained from surgically excised specimens. Chicken breast, yeast and wheat germ phosphoglycerate mutases were prepared as previously described[3–5].

Phosphoglycerate mutase was assayed as previously described[6]. Mutase was measured in the 10 000 × *g* supernatant fraction of 20% water homogenates of human uterus and human skeletal muscle, in saline-washed red cell lysates, and in water extracts of acetone powders from human and pork heart and bovine liver and brain.

For the *in vivo* studies, 2,3-phosphoglycerate phosphatase activity was assayed according to the method of JOYCE AND GRISOLIA[7]. (Phosphate was determined by the GOMORI[8] method.) In other cases phosphatase determinations were made using the radioactive assay of HARKNESS AND PONCE[9]. Radioactive 2,3-[^{32}P]diphosphoglycerate was prepared as previously described[10].

RESULTS

Phosphoglycerate mutase and 2,3-diphosphoglycerate phosphatase activities

TABLE I

EFFECT OF Hg^{2+} AND DITHIOERYTHRITOL ON MUTASE AND PHOSPHATASE ACTIVITY OF RAT KIDNEY

Each tube contained 1 ml of supernatant fraction from 10% homogenates (controls). When indicated, 1 μmole of $HgCl_2$ in 0.05 M Tris–HCl buffer (pH 7.3) was added and the samples incubated for 5 min at room temperature prior to assay. Similarly, 0.05 ml of 0.1 M dithioerythritol was added to samples treated with Hg^{2+} and after 5 min the samples were reassayed. The mutase and phosphatase activities were determined as previously described[3,4].

	Number of animals	*Mutase**	*Phosphatase***
Control	13	47 ± 3.8	400 ± 32
Hg^{2+} added	3	35 ± 4.2	360 ± 46
Dithioerythritol added	3	42 ± 4.0	280 ± 56

* Mean units/ml of homogenate.
** Mean nmoles of phosphate liberated per ml of homogenate; incubation time 30 min.

of crude rat kidney preparations were both minimally affected by treatment with Hg^{2+} as shown in Table I. This finding is in contrast to the previously described reversible Hg^{2+}-induced inhibition of mutase and stimulation of 2,3-diphosphoglycerate phosphatase activities of phosphoglycerate mutase from chicken and rabbit muscle[2]. Obviously, this represents a different behavior of the phosphoglycerate mutase from kidney. However, since there are many nonspecific phosphatases in crude homogenates, the possibility that there was an effect on phosphatase that may have been obscured could not be excluded.

Table II illustrated that the intraperitoneal injection of $HgCl_2$ at very high doses does not influence the phosphoglycerate mutase or the 2,3-diphosphoglycerate phosphatase activities of rat kidney homogenates. It was, therefore, of interest to determine if this lack of effects both *in vivo* and *in vitro* reflected an intrinsic difference between the phosphoglyceromutase from these tissues and that from other animal tissues or the fact that the kidney possesses many phosphatases and a relatively low

TABLE II

EFFECT OF PARENTERAL ADMINISTRATION OF $HgCl_2$ ON THE MUTASE AND PHOSPHATASE ACTIVITY OF KIDNEY HOMOGENATES

500 μg of $HgCl_2$ dissolved in 1.0 ml of phosphate buffer (pH 7.4) were injected intraperitoneally into rats. After the indicated time, the rats were killed and the mutase and phosphatase activities tested. Data expressed are the mean values as described in Table I for each group. When indicated, 0.05 ml of a 0.1 M dithioerythritol solution was added to 1 ml of water homogenate and the preparation assayed after 5 min incubation.

Number of rats	*$HgCl_2$ injected (μg)*	*Intoxication period days*	*Mutase (mean)*		*Phosphatase (mean)*	
			No dithioerythritol	*Dithioerythritol*	*No dithioerythritol*	*Dithioerythritol*
6	500	1	51	47	270	340
3	500	2	33		130	178
3	500	3	36		300	330
3	500	4	43		380	

concentration of mutase which then would mask the effect of Hg^{2+}. Therefore, attempts were made to partially purify the dog and rat kidney mutase.

Partially purified mutase from dog and rat kidney was prepared as follows: 20% water homogenates were made. After centrifugation at 20 000 x *g* for 10 min, the supernatant was mixed with 1.2 vol. of $(NH_4)_2SO_4$ and then centrifuged for 10 min. The precipitate was discarded. $(NH_4)_2SO_4$ (1.8 vol.) was added to the supernatant. The precipitate was taken up in water to approx. 1/10 of the original volume. This partially purified fraction yielded about 70% of the original mutase activity.

TABLE III

EFFECT OF Hg^{2+} ON THE MUTASE ACTIVITY OF DOG AND RAT KIDNEYS AND CHICKEN BREAST

Each tube contained 200 μmoles of Tris–HCl buffer (pH 7) and designated amounts of $HgCl_2$ and mutase in a total volume of 1 ml. Rat and dog kidney mutase consisted of partially purified fractions as described in the text; chicken breast mutase was prepared as previously described[3]. The combination of dog + chicken consisted of equal units of mutase from each preparation. Details of the Hg^{2+} treatment and mutase assay are given in the legend to Table I.

Hg^{2+} added (μmoles)	*Mutase (units)*			
	Dog	*Chicken*	*Dog + chicken*	*Rat*
0	58.5	60	130	75.0
1	53.0	0	53	62.5
2	53.0	0	53	72.5
3	52.0	0	53	75.0

Partially purified preparations of rat and dog kidney mutase as well as a preparation of chicken muscle mutase were treated with Hg^{2+}. As shown in Table III, Hg^{2+} did not affect the mutase activity of dog and rat kidney preparations but decreased the activity of chicken muscle mutase. When the preparations from dog kidney and chicken muscle were mixed and then Hg^{2+} added, the activity decreased in proportion to the calculated inhibition of the muscle enzyme, demonstrating that the presence of other proteins which could bind Hg^{2+} in the crude kidney enzyme preparation were not responsible for the lack of effect with Hg^{2+}. Again for purposes of comparison, Table III shows that mutase from rat kidney is not inhibited by Hg^{2+}.

To confirm the above findings, and in view of the relatively low phosphatase activity of mutase preparations (as a dual function), it seemed important to extensively purify mutase from kidney. Fresh pork kidneys were obtained from a local slaughterhouse. Gross fat was removed and the tissue was ground with a large meat grinder. 2-kg portions of ground tissue were homogenized in 800 ml of water in a large Waring blender for 30 sec. Usually 7–8 kg of ground tissue were processed in a single preparation. The homogenized tissue was poured with vigorous stirring into 10 vol. of cold acetone. After the tissue settled, the bulk of acetone was decanted and the precipitate was dried under suction on two large Büchner funnels covered with rubber dams. After 2 h, the nearly dried cakes were pulverized with the aid of a vegetable grater and sifted (under nitrogen) through a set of mechanically shaken screens of decreasing size. The resultant fine powder was finally dried in desiccators over alumina.

200 g of acetone powder were suspended in 10 vol. of water, stirred for 20 min and then centrifuged at 7000 × *g* for 10 min. The supernatant is the Crude Fraction. 1.6 vol. of 95% ethanol were added to the Crude Fraction and the mixture centrifuged at 5000 × *g* for 15 min. Another 1.6 vol. of ethanol were added to the supernatant. After centrifugation, the precipitate was transferred, with thorough mixing, into 50 ml of water, centrifuged for 30 min at 15 000 × *g* and any insoluble material discarded. The supernatant (Ethanol Fraction) was diluted to a protein concentration of 12 mg/ml after the addition of 0.05 vol. of 0.1 M 2,3-diphosphoglycerate. The mixture was brought to 54° in a water bath and held at that temperature for 4 min. After cooling in an icebath it was centrifuged at 10 000 × *g* for 15 min. In practice the supernatants (Heated Fraction) from three 200-g acetone powder batches were pooled at this stage. 1.5 vol. of $(NH_4)_2SO_4$ were added to the Heated Fraction and the mixture was centrifuged at 6000 × *g* for 20 min. 5 vol. of $(NH_4)_2SO_4$ were added to the supernatant and the fraction was centrifuged at 6000 × *g* for 30 min. The loosely packed precipitate was transferred to a 40-ml centrifuge tube and centrifuged at 15 000 × *g* for 30 min. This well-packed pellet was taken up in a minimum volume of water (approx. 10 ml; protein concentration approx. 35 mg/ml). The fraction was centrifuged at 15 000 × *g* to remove insoluble material and the supernatant $(NH_4)_2SO_4$ Fraction) was layered on a 2.8 cm × 120 cm column of Sephadex G-100, equilibrated with 0.02 M sodium phosphate buffer (pH 7). The column was eluted with equilibrating buffer at a flow rate of approx. 10 ml/h; 3-ml aliquots were collected. Two major protein peaks were eluted, the first eluting at the void volume for the column. The mutase activity appears at 1.1–1.2 volumes. The tubes containing mutase activity with a specific activity above 250 were pooled (Sephadex Fraction). This fraction was percolated through a 2.5 cm × 50 cm column of DEAE-cellulose, equilibrated with 0.02 M sodium phosphate (pH 7). The column was washed with 50 ml of equilibrating buffer. The column was developed utilizing a linear gradient consisting of 1000 ml of the 0.2 M Na_2HPO_4 in the reservoir vessel and 1000 ml of the 0.02 M equilibrating buffer in the mixing chamber. Flow rate was maintained at 20 ml/h. The tubes were assayed for protein and mutase activity. Three major protein peaks were eluted in a reproducible fashion; the third protein peak contained the mutase activity. Tubes in the latter peak containing mutase with a specific activity above 1200 were pooled (DEAE-Cellulose Fraction). This fraction was concentrated to approx. 5 ml in a Model 50 Diaflo ultrafiltration assembly (Amicon Corporation) equipped with a PM-10 membrane. 10 vol. of 0.01 M sodium phosphate (pH 7) were added to the fraction in the chamber and the fraction was then concentrated to approx. 10 mg/ml. This fraction is stored frozen until further use. A summary of the purification procedure is given in Table IV.

Gel filtration estimates of the molecular weight, using Sephadex G-100 as described by ANDREWS[11], revealed a molecular weight of about 65 000 for the purified kidney mutase. The elution volume was almost identical to that of purified bovine plasma albumin (Pentex Corporation) which served as one of the standard molecular weight markers.

The final fraction possessed a specific activity approximating that observed with phosphoglycerate mutase purified from chicken breast[3], rabbit muscle[6] and yeast[4]. However, acrylamide gel disc electrophoresis[12] demonstrated four separate bands of protein in the final fraction. Mutase activity was present in all four of these bands

TABLE IV

SUMMARY OF PURIFICATION

Fraction	*Vol. (ml)*	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Recovery** (%)*
Crude	1550	127 100	22 630	5.6	100
Ethanol	68	72 080	1 360	53	56
Heated	98	59 000	588	122	46
$(NH_4)_2SO_4$*	12	120 000	636	190	31
Sephadex pool*	43	110 000	210	522	29
DEAE-cellulose pool*	17	35 000	20.6	1730	9

* Data given for three 200 g batches of acetone powder.

** Recovery calculated from crude acetone powder extract; this fraction contains approx. 80% of the mutase activity of the original tissue and about 6-fold increase in specific activity.

when the corresponding areas of unstained paired gel columns were extracted by freeze–thawing in 1 vol. of water subunit aggregation (dimers, tetramers, *etc.*) was suggested by these findings. Since completion of this work, the existence of six isoenzymes for chicken muscle phosphoglycerate mutase has been reported[13].

The effect of Hg^{2+} on purified kidney mutase and its 2,3-diphosphoglycerate phosphatase activity is shown in Table V. It is apparent from this data that Hg^{2+} exhibited no appreciable effect on either activity.

TABLE V

THE INFLUENCE OF $HgCl_2$ ON KIDNEY PHOSPHOGLYCERATE MUTASE

The effect of Hg^{2+} on mutase activity was determined as follows: designated amounts of Hg^{2+} were added to tubes containing 0.1 ml of enzyme (2.6 mg/ml, specific activity 1500) and 50 mM Tris–HCl (pH 7.0) in a total volume of 0.2 ml. After 5 min at 37°, 1:100 dilutions with 1 mg/ml bovine serum albumin were assayed for mutase activity as previously described[3]. For the phosphatase assay, 0.5 μmole of Hg^{2+} was added to tubes containing 50 μl of enzyme fraction and 100 mM Tris–acetate (pH 6.5) in a total volume of 0.25 ml. After 5 min at 37°, 0.1 μmole of 2,3-[$^{32}P_2$]diphosphoglycerate was added to each tube and the procedure of HARKNESS AND PONCE[9] was followed employing a 60-min incubation.

$HgCl_2$ (mM)	*% of initial activity after Hg^{2+} treatment*	
	Mutase	*Phosphatase*
0	100	100
0.5	105	99
1.0	116	98
2.0	110	99
5.0	86	

The effects of Hg^{2+} upon phosphoglycerate mutase (and its 2,3-diphosphoglycerate phosphatase activity) from a number of additional sources was examined. The results, summarized in Table VI, demonstrate again no effect of Hg^{2+} on either activity from the purified kidney preparation. However, a striking inhibition of mutase activity and simultaneous stimulation of phosphatase activity of human skeletal muscle and of purified chicken breast phosphoglycerate mutase was observed

TABLE VI

EFFECT OF $HgCl_2$ ON PHOSPHOGLYCERATE MUTASE FROM SEVERAL TISSUES

Details of experimental conditions are given in Table V. The concentration of $HgCl_2$ was 2 mM in all cases.

Source	*% of initial activity after Hg^{2+} treatment*	
	Mutase	*Phosphatase*
Pork kidney, purified	110	99
Pork kidney, crude	58	55
Chicken breast	1	178
Chicken breast, crude	7	130
Human skeletal muscle	6	154
Human heart	7	56
Pig heart	31	48
Human uterus	50	54
Red cell	80	62
Beef liver	68	93
Calf brain	88	50
Human marrow	155	

after Hg^{2+} treatment. The phosphoglycerate mutase as well as the 2,3-diphosphoglycerate phosphatase activities of human uterus, human and porcine cardiac muscle were both significantly inhibited by Hg^{2+} treatment.

In view of the demonstrated differences in behavior of both the mutase and phosphatase activities in several tissues, additional studies were carried out to compare the effects of phosphoglycolate, bisulfite and pyrophosphate upon the phosphatase activities of these same enzyme preparations. All of these reagents have been reported to stimulate 2,3-diphosphoglycerate phosphatase activity[9,14–16]. For compactness of presentation, only the data at the optimal concentration of the effectors is given. The results are summarized in Table VII. Phosphatase activity from all

TABLE VII

EFFECT OF SEVERAL REAGENTS ON 2,3-DIPHOSPHOGLYCERATE PHOSPHATASE ACTIVITY OF SEVERAL PHOSPHOGLYCEROMUTASES

Source	*% of initial activity after treatment*			
	Bisulfite (20 mM)	*Pyrophosphate (20 mM)*	*Phosphoglycolate (2 mM)*	*$HgCl_2$ (2 mM)*
Pork kidney, purified	150	192	446	99
Pork kidney, (crude)	50	113	473	55
Chicken breast	29	245	317	178
Yeast	115	95	106	80
Human skeletal muscle	51	709	860	154
Human heart	45	113	440	42
Pork heart	38	66	185	48
Human red cells	226	242	4150	62
Beef liver	89	252	373	93
Beef brain	60	104	180	50
Human uterus	92	455	734	54

sources was stimulated by phosphoglycolate but particularly in the case of the red cell. Pyrophosphate stimulated the 2,3-diphosphoglycerate phosphatase activity of all mutase preparations, particularly that of human skeletal muscle, but to a lesser extent than phosphoglycolate. Bisulfite significantly inhibited 2,3-diphosphoglycerate phosphatase activity of the chicken breast, heart muscle and human skeletal muscle preparations and, on the other hand, stimulated the phosphatase of pork kidney mutase and red cells. The phosphatase activity of yeast mutase was little affected by any of the reagents. Phosphoglycerate mutase activity was unaffected by these reagents.

DISCUSSION

The procedure described for purifying pork kidney phosphoglycerate mutase some 2000-fold from the tissue levels is very reproducible but not as simple and much more time consuming than procedures described for the enzyme from other sources[3,4,6]. The phosphoglycerate mutase activity of crude homogenates of pork kidney proved very difficult to purify until acetone powders of fresh or frozen tissue were prepared. For example, the phosphoglycerate mutase activity of freshly prepared homogenates progressively decreased at 4°, reaching approx. 25% of initial activity after 8 h. Similarly, all attempts to fractionate crude homogenates with solvents, including acetone, ethanol and methanol at 0°, were unsuccessful because of rapid and complete enzyme inactivation by these reagents. The crude enzyme was rapidly inactivated at pH values below 5,5, precluding the use of commonly used protein precipitating agent. Phosphoglycerate mutase activity of crude homogenates was also extremely heat sensitive, showing extensive inactivation at 45–50°. Preparation of an acetone powder proved to be the critical step in enzyme purification. Quantitative recovery of phosphoglycerate mutase present in fresh tissues was achieved. The enzyme now was stable indefinitely as a powder. The water extract of this acetone powder contained 75–80% of the original kidney mutase activity; the specific activity of this extract was 5–6 compared to <1 for fresh homogenates of kidney. Equally important for further purification, the phosphoglycerate mutase activity of the acetone powder extracts was stable at 4° for at least 48 h, was stable in the presence of ethanol and acetone at 4° allowing fractionation, and was sufficiently stable to heat to allow the use of a heat step in purification.

Extensive purification of the enzyme has made it possible to study a number of parameters and to clarify the effect of Hg^{2+}. Certainly it would appear from these findings that the effects of Hg^{2+} in lower nephron nephrosis cannot be explained by an effect on phosphoglycerate mutase. The molecular weight estimate and specific activity are in keeping with previously reported studies on phosphoglycerate mutases[3,8,11].

Phosphoglycerate mutase from kidney is shown to be most resistant to Hg^{2+} treatment as judged by both parenteral administration of Hg^{2+} to rats and by *in vitro* addition of Hg^{2+} to crude as well as purified kidney mutase preparations. Similarly, the 2,3-diphosphoglycerate phosphatase activity of purified pork kidney mutase is equally resistant to Hg^{2+} treatment. However, human skeletal muscle phosphoglycerate mutase demonstrated inhibition of mutase and stimulation of 2,3-diphosphoglycerate phosphatase activity after Hg^{2+} treatment similar to that observed

with purified chicken or rabbit muscle phosphoglycerate mutase[2]. Crude preparations of phosphoglycerate mutase from beef brain and liver and human red cells behaved in similar fashion to kidney mutase after Hg^{2+} treatment. The present as well as previous studies[2] showed heart muscle phosphoglycerate mutase (human and pig) to be more sensitive to Hg^{2+} treatment, while the accompanying 2,3-diphosphoglycerate phosphatase activity was less sensitive to Hg^{2+} than was the kidney enzyme. However, stimulation of phosphatase activity by Hg^{2+} was not observed in these studies. The stimulation of phosphatase activity by Hg^{2+} previously reported was in large part due to nonenzymatic hydrolysis of 2,3-diphosphoglycerate exhibited by $HgCl_2$; thus, small changes in total phosphatase activity were in some cases misleading.

As discussed by DIXON AND WEBB[17], the presence of two enzymes catalyzing the same reaction in different tissues implies the presence of two genetic loci. Alternatively one may consider the existence of an enzyme which may modify the SH groups of phosphoglyceromutase in a particular tissue. Of course, the simpler cases of lack of appearance of an enzyme indicate the existence of a repressor, as may be the case for hexokinases with low K_m which change with diet, age, *etc.*[18] It is not clear, however, why different tissues sometimes manifest different proportions of different enzymes such as the transaminases. The best-known example of a different enzyme catalyzing the same reaction in different tissues of a single species is phosphorylase, *i.e.* muscle and liver[19]. Nevertheless, to the knowledge of the writers there is no case of differences in tissues as is described here for the behavior to Hg^{2+} of phosphoglyceromutase. It may be exceedingly important to find out if there is a relation between distribution and/or changes in expression of the Hg^{2+}-sensitive and -insensitive enzymes during development.

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