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THE EFFECT OF THYROID HORMONES AND OTHER KINETIC MODIFIERS ON BISPHOSPHOGLYCEROMUTASE FROM HUMAN ERYTHROCYTES

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Abstract—Bisphosphoglyceromutase (E.C. 2.7.5.4) is inhibited by thyroxine, tri-iodothyronine and di-iodothyronine and activated by glucagon. Several other hormones have no effect. The enzyme is activated by various thiols, but not by NADH, NADPH or ascorbic acid. The inhibition by α- and β-glycerophosphate and the activation by phosphoglycollic acid [1] have been confirmed. 2-Hydroxy-4-phosphobutyric acid, an isostere of β-glycerophosphate, has no effect on the kinetics. Nicotine is slightly inhibitory at concentrations much higher than the plasma level in smokers.

As early as 1964, Tapley [2] documented the effect of thyroxine and tri-iodothyronine on over 50 enzymes. Numerous studies since that time have appended this category of enzymes both *in vivo* and *in vitro*. Although the mechanism of action of the thyroid hormones remains unknown, their diversity of action suggests that effective structural analogues of the iodothyronines would have a wide range of possible pharmacological applications.

The effects of hormones and other kinetic modifiers on the enzymes of the Rapoport–Luebering shunt have not received much study and some of the reported results disagree. Thus Snyder, Reddy and their co-workers [3–6] reported that thyroxine and tri-iodothyronine increase the amount of 2,3-bisphosphoglycerate in the erythrocytes as a direct consequence of the activation of bisphosphoglyceromutase (E.C. 2.7.5.4). These results were disputed by others [7–10]. Somewhat earlier, it had been reported [11] that thyroxine in the range 0.5–100 μM, when preincubated with bisphosphoglyceromutase from chicken breast muscle, caused substantial inhibition.

There is also conflicting evidence on the influence of tri-iodothyronine from experiments *in vivo*. Injection of rats with tri-iodothyronine (20 μg/100 g body weight) during 10 days did not affect the level of 2,3-bisphosphoglycerate [12], whereas similar treatment of rats on a atherogenic diet caused an increase in the level of 2,3-bisphosphoglycerate in the erythrocytes [13].

The purpose of the work reported here was to study in some detail the effect of thyroid hormones and several other hormones on the steady-state kinetics of bisphosphoglyceromutase. In addition, the behaviour of the enzyme in the presence of other potential kinetic modifiers including thiols and nicotine was examined.

MATERIALS AND METHODS

Purified bisphosphoglyceromutase was prepared from outdated human blood [2]. 1,3[1-^{32}P] Bisphosphoglycerate was prepared from DL-glyceraldehyde 3-phosphate [1] and the product was assayed spectrophotometrically [14]. 2-Hydroxy-4-phosphonobutyric acid [15] was a gift from Dr. H. B. F. Dixon. Porcine glucagon was a gift from Professor K.D. Buchanan.

Kinetic methods. Procedures for assaying 2,3-bisphosphoglyceromutase using 1,3-[1-^{32}P] bisphosphoglycerate were based on those of Rose [1]. As a screening method to determine the effect of hormones and other modifiers, the following procedure was employed: Bisphosphoglyceromutase (2.2×10^{-5} U) was incubated in 17.5 mM glycylglycine–HCl buffer (0.1 ml), pH 7.8, which was 2 mM with respect to 2-mercaptoethanol for 30 min at 26°. NaOH, 2 mM (0.2 ml), alone or containing the modifier under test, was added and the incubation was carried out for a further 30 min. A buffer, pH 7.5 (0.2 ml), containing 41.5 mM glycylglycine, 50 mM K_2HPO_4, 1.5 mM 2-mercaptoethanol and either 50 μM 3-phosphoglycerate or 50 μM 2-phosphoglycerate was added and the mixture was incubated for a further 30 min. 1,3-[1-^{32}P] Bisphosphoglycerate solution, 0.8 μM (0.5 ml), was added to give a final volume of 1.0 ml and aliquots (0.2 ml) were removed after 0.5, 6.0, 12.0 and 18.0 min for the determination of the radioactive product. Each such aliquot was placed in a separate plastic tube (75 × 12 mm) containing 2 M H_2SO_4 (0.1 ml). The hydrolysis of the unreacted 1,3-bisphosphoglycerate was catalysed by the addition of 5% (w/v) ammonium molybdate (0.1 ml), then water (0.6 ml) and water-saturated isobutanol (2 ml) were added. The tubes were mixed vigorously and centrifuged (1200 *g*, 5 min). The upper layer contained the phosphate from the unreacted and subsequently hydrolysed substrate extracted as phosphomolybdate in the isobutanol. An aliquot of the lower layer (1 ml) containing the acid-stable labelled 2,3-bisphosphoglycerate was assayed for radioactivity by liquid scintillation counting. In a separate experiment it was shown that the count-rate was proportional to the amount of 2,3-bisphosphoglycerate contained in the lower layer.

When this screening method was used to investigate the effects of reducing agents, 2-mercaptoethanol was omitted from the buffer used for the first incubation, i.e. 16 mM glycylglycine–HCl buffer, pH 7.8. For scintillation counting of aqueous samples (0.5–1.0 ml), solutions containing 0.5% (w/v) PPO and 0.03% (w/v) 1,4 bis-2-(4-methyl-5-phenyloxazoylyl)-benzene in toluene followed by ethanol (7 ml) were added. Counting was carried out with a Packard Tricarb 3003 instrument. Chemiluminescence was observed, especially with standard solutions of 1,3-[1-^{32}P]-bisphosphoglycerate. This complication was avoided by preparing the standard solutions in 0.2 M H_2SO_4. Both standard and test solutions were kept in the dark at 0° for 24 hr before counting.

For the determination of K_m and V_{max}, the bisphosphoglyceromutase was pre-incubated with iodothyronine for 30 min, 9 concentrations (0.1–4.0 μM) of substrate were used and each of these sampled 7 times from 30 sec to 18 min. For these kinetic experiments only, a second isobutanol extraction was used.

Initial velocities, K_m and V_{max} were computed using FORTRAN programmes based on published statistical procedures [16]. The initial estimates of K_m and V_{max} were refined by an interactive non-linear weighted least squares regression method [17].

For the determination of the effect of various reducing agents upon the time-course dependence of the bisphosphoglyceromutase reaction, an experimental method was devised in which 4 concentrations of a reducing agent could be tested concurrently.

The steroids which were tested as kinetic modifiers were dissolved in either ethanol or dioxan and a small aliquot was added to the reaction mixture such that the final concentration of organic solvent did not exceed 1.25% (v/v). Control experiments showed that this concentration of organic solvent did not affect the kinetics of the system.

RESULTS AND DISCUSSION

Using the screening method, the iodothyronines were found to be inhibitory over a wide range of concentrations (see Fig. 1).

Tri-iodothyronine proved to be the most potent inhibitor, diodothyronine was least effective and thyroxine had intermediate activity. The effect of fixed concentrations of iodothyronine on the K_m and V_{max} values are summarized in Table 1. With either monophosphoglycerate as cofactor, the K_m value tended to increase and the V_{max} tended to decrease with increasing concentration of iodothyronine. The inhibition appeared to be of the mixed type. When the slopes and ordinal intercepts from weighted least-squares Lineweaver–Burk plots were replotted against inhibitor concentration, the resulting secondary plots were linear. These results, therefore, based on the kinetic experiments as exemplified by Fig. 1 and the results shown in Table 1, contrast sharply with those of Snyder, Reddy and their colleagues [3–6]. On the other hand, the results lend support to those of Joyce and Grisolia [10] obtained with bisphosphoglyceromutase from chicken breast muscle, and other workers who found no activation of erythrocyte bisphosphoglyceromutase by the thyroxine and tri-iodothyronine at low concentrations. Since inhibition was observed only with iodothyronine concentrations above about 10 μM, it is doubtful if the effects of the iodothyronines on the kinetics of the action of bisphosphoglyceromutase have any physiological significance.

Table 1. The effect of inhibitor concentration upon Michaelis constant (K_m) and maximum velocity (V_{max})

(a) In the presence of 10 μM 3-phosphoglycerate as cofactor

Modifier	Concentration (μM)	10^6 K_m ± S.D.	10^9 V_{max} ± S.D.
None	—	1.05 ± 0.04 (18)	7.68 ± 0.18
Thyroxine	12.5	1.14 ± 0.06 (9)	6.20 ± 0.15
Thyroxine	25.0	1.40 ± 0.05 (9)	5.60 ± 0.11
Thyroxine	50.0	1.69 ± 0.07 (18)	4.97 ± 0.13
Thyroxine	75.0	1.99 ± 0.12 (9)	3.85 ± 0.13
Tri-iodothyronine	12.5	1.47 ± 0.07 (9)	7.00 ± 0.20
Tri-iodothyronine	25.0	2.72 ± 0.10 (9)	6.46 ± 0.18

(b) In the presence of 10 μM 2-phosphoglycerate as cofactor

Modifier	Concentration (μM)	10^6 K_m ± S.D.	10^9 V_{max} ± S.D.
None	—	0.99 ± 0.06 (18)	12.10 ± 0.40
Thyroxine	12.5	1.29 ± 0.10 (9)	9.92 ± 0.42
Thyroxine	25.0	1.34 ± 0.05 (9)	9.06 ± 0.21
Thyroxine	50.0	1.46 ± 0.07 (18)	7.42 ± 0.24
Thyroxine	75.0	1.76 ± 0.06 (9)	6.01 ± 0.16
Tri-iodothyronine	12.5	1.64 ± 0.07 (9)	11.70 ± 0.30
Tri-iodothyronine	25.0	3.04 ± 0.13 (9)	9.01 ± 0.28

The figures in parentheses give the number of runs performed to determine K_m and V_{max} with substrate concentrations in the range 0.1–4.0 μM and with $E_o = 1.94 \times 10^6$ units/ml of reaction mixture. One unit of enzyme activity converts one μmole of 1,3-bisphosphoglycerate per min at 25° under the conditions of the radioactive assay.

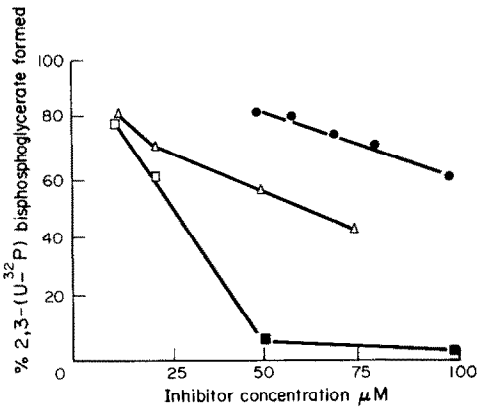

Fig. 1. The inhibition of bisphosphoglyceromutase activity by the iodothyronines. 1,3-[1-^{32}P]-Bisphosphoglycerate (2.4 μM) was the substrate. The data from 9 experiments were normalized. Closed symbols represent results from single substrate concentration experiments. ●, Di-iodothyronine; △, Thyroxine; □, ■, Tri-iodothyronine.

It seems possible that structural analogues of thyroxine and tri-iodothyronine could be effective at pharmacological levels for inhibiting 2,3 bisphosphoglycerate production. Such compounds might be expected to offer a number of interesting possible applications, including control of the equilibrium between deoxy- and oxy-haemoglobin. For example, in patients with the haemoglobinopathy known as Hb. Kansas, a fall in the 2,3 bisphosphoglycerate level would cause a shift to the oxy-form which in this condition is inherently less stable than the deoxy-form. Thus a shift of this type would enhance the oxygen carrying capacity of these patients. Since thyroxine and tri-iodothyronine enhance the activity of certain enzymes while inhibiting the activity of others, pharmacologically effective analogues of the iodothyronine could also be expected to find application in controlling the rate of many other enzymic reactions. It is noteworthy that thyroid hormones at pharmacological levels stimulate transport of amino acids across cellular membranes (reviewed by Sterling [18]). Consequently, analogues of the iodothyronines might display anabolic activity without the side effects found with androgens.

Using the screening method, both α-glycerophosphate and β-glycerophosphate were found to be inhibitory, causing 50% and 5% inhibition, respectively, at a concentration of 200 μM with initial substrate and 3-phosphoglycerate concentrations of 0.4 and 2.0 μM, respectively. Although the degree of inhibition caused by β-glycerophosphate is small and probably close to the experimental error, nevertheless a small difference was consistently observed between runs carried out in its presence and absence. In contrast, 60 μM phosphoglycollic acid caused 260% activation with initial substrate and 3-phosphoglycerate concentrations of 0.4 and 0.18 μM, respectively. These results are in general agreement with those reported in Rose [1].

2-Hydroxy-4-phosphonobutyric acid (0.1 mM) [15], an isostere of β-glycerophosphate, had no effect. Other compounds which had no effect included aldosterone (0.005–50 μM), dehydroepiandrosterone (0.01–100 μM), hydrocortisone (0.0067–67 μM), insulin (0.0013–13 μM), DL-noradrenalin (0.0022–22 μM), 17 β-oestradiol (0.005–50 μM) and testosterone (0.005–50 μM). Glucagon was found to be an activator causing an acceleration of 10% at 0.44 μM and 25% at 4.4 μM with initial substrate and 3-phosphoglycerate concentrations of 0.4 and 10 μM, respectively. The activation by glucagon is probably insignificant *in vivo* since it is unlikely to enter the red cell. The effect of nicotine was tested over the concentration range 0–4 mM, with substrate concentrations of 4.0 and 10 μM, respectively. The inhibitory effect is unlikely to be physiologically significant since the plasma level of nicotine in smokers is probably less than 1 μM [19].

Highly purified bisphosphoglyceromutase requires the addition of a reducing agent for optimal activity. In order to ascertain whether other reducing agents, particularly those of the thiol type, could substitute for 2-mercaptoethanol in enhancing the activity of the purified enzyme, two experimental procedures were employed. In the first, the effects of various reducing agents on the time-course of the bisphosphoglyceromutase reaction were determined. Cysteine, thioglycollic acid and 3-mercaptopropionic acid gave similar results to those obtained with 2-mercaptoethanol. The degree of enhancement was relatively greater at the beginning of a given incubation period than towards the end of it. In the second type of experiment, the enzyme was preincubated with various reducing agents as described under Materials and Methods. The following compounds were tested at a concentration of 1 mM and the initial velocities compared with those measured in the presence of 1 mM, 1,4-dithiothreitol are given in parenthesis: 2-mercaptoethanol (65%); L-cysteine (79%); dimercaptopropanol (65%); thioglycollic acid (65%); 3-mercaptopropionic acid (87%) and reduced glutathione (62%).

It is likely that glutathione, which is probably about 2 mM in the red cell [20], is the natural activator. It is possible that the enzyme contains a thiol group at or near the active centre which tends to undergo oxidation to disulphide in the absence of a protective thiol such as glutathione. NADH, NADPH and ascorbic acid were also tested, but had no effect on the kinetics.

2,3-Bisphosphoglycerate plays several roles in the metabolism of erythrocytes. As an allosteric effector, it is partly responsible for controlling the equilibrium between deoxyhaemoglobin and haemoglobin [21–23]. There is evidence that 2,3-bisphosphoglycerate exerts some control on glycolysis by inhibiting hexokinase [24–27], phosphoglucomutase [26], 6-phosphofructokinase [26–28], glyceraldehyde–phosphate dehydrogenase [26] and pyruvate kinase [28], as well as inhibiting its own production by bisphosphoglyceromutase [1]. 2,3-Bisphosphoglycerate also helps to conserve adenine nucleotides in the erythrocyte by inhibiting AMP deaminase [29] and ribose phosphate pyrophosphokinase [30].

Consequently, there is considerable interest in identifying the factors which determine the concentration of 2,3-bisphosphoglycerate in the erythrocyte.

It might be expected that the Rapoport–Luebering shunt would contain a control mechanism for 2,3-bisphosphoglycerate turnover, but this remains to be elucidated.

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