RAT LIVER 6-PHOSPHOGLUCONOLACTONASE: A LOW K_m ENZYME

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SUMMARY. By specific enzymic synthesis of the substrate 6-phosphogluconolactone *in situ*, the K_m for rat liver 6-phosphogluconolactonase was found to be 80 μM. This value is approximately 100 fold lower than the previously determined value, and is compatible with the kinetic parameters of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and hence with the flux through the oxidative segment of the pentose phosphate pathway.

The initial, and oxidative, phase of the pentose phosphate pathway involves the two dehydrogenases, G6PDH* and 6PGDH. It might thus be expected that the maximal catalytic activity of both these NADP-specific dehydrogenases would be modulated in a completely synchronous manner. However, Sapag-Hagar *et al.* (1) have demonstrated that, for rat liver, there is an apparent imbalance in the specific activities of the two enzymes under a variety of conditions, and that the specific activity of 6PGDH is much smaller than the corresponding activity of the G6PDH at free $NADP^+$/NADPH ratios in the physiological range (0.1 - 0.01). Since no naturally occurring, specific activators of 6PGDH have been found (unpublished results), the low measured or calculated 6PGDH/G6PDH activity ratio may suggest the possibility of an impairment of flux through the pathway at that site. The immediate product of G6PDH is the lactone of 6PG, *viz*, 6-phosphogluconо-δ-lactone (6PGL), and the hydrolysis of the lactone to the free acid is generally assumed to occur by spontaneous hydrolysis and/or by the action of a specific 6-phosphogluconolactonase. However, little is

*Abbreviations: G6PDH, glucose 6-phosphate dehydrogenase (EC 1.1.1.49); 6PGDH, 6-phosphogluconate dehydrogenase (EC 1.1.1.44); G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconо-δ-lactone.

known about this lactonase in mammalian systems despite the fact that the lactonase provides the link between the two dehydrogenases. For rat liver 6-phosphogluconolactonase, Kagawa *et al.* (2) have obtained a K_m value for the lactone of approx. 7 mM, and a total activity of approx. 35 U/g tissue (3). In view of the respective specific activities and K_m values of the two dehydrogenases, the K_m value of 7 mM for the lactonase seems anomalously high. This communication reports a re-investigation of the basic kinetic parameters of the lactonase.

Experimental. The livers of adult rats fed *ad lib* were homogenised with 4 vols of 5 mM K_2HPO_4-1 mM dithioerythritol-150 mM KCl-1 mM $MgCl_2$-0.5 mM $CaCl_2$, adjusted to pH 7.0. The supernatant resulting from centrifugation at 30,000 g x 30 min at 4° was used immediately or stored overnight in a frozen state.

For kinetic studies the reaction mixture contained, in a total vol of 1 ml, 5 mM $NADP^+$, 100 mM imidazole pH 7.0, approx. 10 U G6PDH and 5 mM $MgCl_2$. The reaction giving 6PGL formation was started by the addition of G6P (conc. range: 30 - 200 µM) and after 30 sec, approx. 2.5 U 6PGDH was added. After a further 30 sec, tissue extract as a source of 6-phosphogluconolactonase was added. The change in extinction at 340 nm was recorded (path length: 1 cm).

For measurement of the spontaneous hydrolysis of 6PGL at 25° and pH 7 and pH 7.8 by the method of Horecker and Smyrniotis (4), the reaction mixture contained, in a total vol of 1 ml, 1.5 mM $NADP^+$, 50 mM Tris (pH 7.0 or 7.8), approx. 5 U G6PDH, 1 U 6PGDH and 5 mM $MgCl_2$. The reaction was started by the addition of 50 µM G6P, and the extinction at 340 nm determined.

Values are quoted as the mean ± S.E.M. The lines of best fit were computed by the method of least squares.

Results and discussion. Major difficulties in the study of the lactonase are the unavailability and lability of the substrate. Preparation of 6PGL by the method of Horecker and Smyrniotis (4) proved unsatisfactory for routine kinetic enzyme studies. In our hands, this method, *viz*, bromine oxidation of G6P, gave low and unreliable yields of 6PGL, despite approximately 80% utilisation of G6P which is similar to the value obtained by Horecker and Smyrniotis (4). The major product was 6PG, the yield of lactone being less than 20%, and experimental manipulations to improve the yield proved unsuccessful. Thus the only feasible method to routinely synthesise the lactone was to synthesise it *in situ* by the use of the appropriate mass of G6P and a relatively large amount of G6PDH, and at a relatively low pH to reduce spontaneous hydrolysis

of the subsequent lactone. The values cited for the half-life of 6PGL are varied, ranging from approx. 1.5 min at pH 7.4 (4) to 20 min at pH 7.0 (5) and 24 min at pH 6.4 (6). By application of the methodology of Horecker and Smyrniotis (4), in which G6P is reacted with a large excess of G6PDH in the presence of 6PGDH, spontaneous hydrolysis of the lactone at 25° was found to follow first order kinetics, the half-life at pH 7.0 and pH 7.8 being approx. 7 and 3.5 min respectively. Thus, at room temp. and at pH 7.0, the preparation of the lactone *in situ* proved feasible.

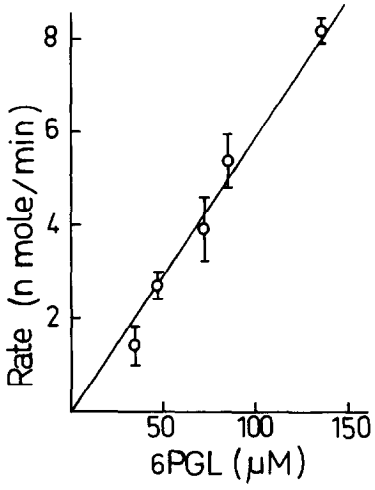

Fig. 1. The rate of spontaneous hydrolysis of the 6-phosphoglucono-δ-lactone formed *in situ* at varying concentrations of lactone. The values are expressed as the mean ± S.E.M. (n = 3). The regression line was computed by the method of least squares ($r = 0.96$, $p < 0.001$).

In the presence of a large excess of G6PDH, 69.8 ± 1.4% of the G6P (conc. range: 30 - 200 μM) was rapidly converted to the lactone. Addition of 6PGDH after 30 sec demonstrated that the rate of spontaneous hydrolysis of the lactone at pH 7.0 was 5.5 ± 0.3% per min over the concentration range used. This rate of spontaneous hydrolysis exhibited first order kinetics (Fig. 1) giving a half-life of 12 min at pH 7.0, which is in good agreement with the half-life as determined by the method described above. Subsequent addition

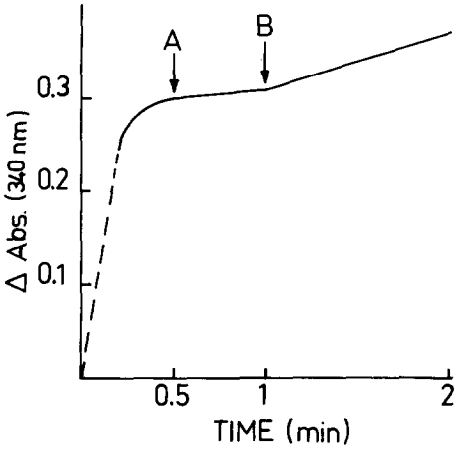

Fig.2. Typical 6-phosphogluconolactonase assay. The reaction mixture contained 5 mM NADP, 100 mM imidazole pH 7.0, approx. 10 U G6PDH and 5 mM $MgCl_2$. The reaction was started by the addition of G6P (65 μM). After 30 sec approx. 2.5 U 6PGDH was added (A) to determine the rate of spontaneous hydrolysis of the lactone. After a further 30 sec tissue extract (10 μl) was added (B) and the initial velocity of the lactonase determined.

of tissue extract caused rapid hydrolysis as manifested in the increase in absorption at 340 nm due to reduction of $NADP^+$ which, in turn, was due to oxidation of the 6PG formed by the endogenous lactonase (Fig.2). This method was routinely used for the kinetic studies.

Fresh tissue extracts tended to give erratic values for the K_m value for the lactone, but suggested that the value was quite low. Storage of the extract overnight in a frozen state resulted in less than 10% loss in activity, and the thawed extract showed less variability of the K_m value. Under these conditions, the K_m for the lactone was found to be 80 μM, with maximal activity in the normal animal of 10 U/g tissue (Fig.3). The total activity is thus similar to that found by Kawada *et al.* (3), but the K_m value is two orders of magnitude less than that determined by Kagawa *et al.* (2). However, it should be noted that Kagawa *et al.* (2) measured the disappearance of lactone by the non-specific oxamate method and this method is sensitive only in the millimolar range; that the pH of the incubation medium was not

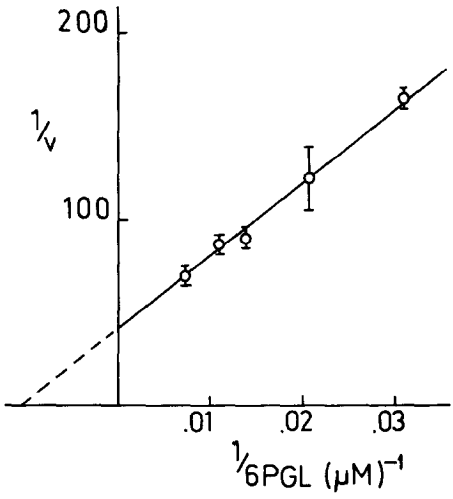

Fig.3. Lineweaver-Burk plot with 6-phosphogluconolactone as the substrate synthesised *in situ*. The reciprocal of the initial velocity is expressed as $(\mu mole/min)^{-1}$. The regression line was computed by the method of least squares ($r = 0.99$, $p < 0.001$), and values are expressed as the mean ± S.E.M. ($n = 3$).

specified, and that the authors stated "it was difficult to keep the exact lineality of the reaction velocity during the assay". Hence, it would appear that the high K_m value obtained by Kagawa *et al.* (2) is a result of experimental artifact due to the non-specific and relatively insensitive method of determination. Kawada *et al.* (3), using the oxamate method, found that "heat treatment at 61^0 for 3 minutes of rat liver supernatant resulted in a marked reduction in lactonase activity". With the specific method of analysis outlined above, we have determined that heat treatment of the extract at 60^0 for 5 min caused 30% loss in activity, whereas treatment for 5 min at 65^0 caused complete loss of lactonase activity.

The use of pH 7.0, within the physiological range, is a satisfactory compromise, since at higher pH values the spontaneous hydrolysis of lactone assumes much greater importance, while at lower pH values the formation of lactone from G6P by G6PDH is hindered by the displacement of the equilibrium, as is the oxidation of the free 6PG by 6PGDH. The determined K_m value of

80 μM for the lactone, and the total lactonase activity of 10 U/g, fit more closely the kinetic parameters of the two dehydrogenases than does the previously determined K_m value of 7 mM. The K_m values of the two dehydrogenases for the respective hexose phosphate substrates are in the range 30-70 μM, and the total activity in the range 1.5-30 U/g over a variety of physiological conditions. Hence, since both the total activity and the K_m of the lactonase are of the same orders of magnitude as the two dehydrogenases and the lactonase is presumably not hindered by the normal accumulation of NADPH, it is unlikely that the lactonase itself would impair the flux between the two dehydrogenases *in vivo*, whereas such an impairment may prevail if the K_m for the lactonase was truly two orders of magnitude greater.

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