The Construction and Testing of a Simple, Slow Delivery–Rapid Quench Apparatus†

William J. Ray, Jr.,*,‡ and James W. Long§

ABSTRACT: A simple, inexpensive, slow delivery–rapid quench apparatus is described. The apparatus can be used to mix small volumes (about 50 μl) of equilibrium mixtures of enzyme-substrate and enzyme-product complexes with a quenching solution, ideally to inactivate the enzyme more rapidly than such complexes can be interconverted. The efficiency of the apparatus is tested by (a) injecting basic solutions of an indicator dye into acid and observing the length of the unbleached plume of dye produced at the delivery tip and (b) by forming an enzyme-substrate complex with ^{32}P-labeled phosphoglucomutase and measuring the extent of label transfer prior to inactivation by the quenching solution. Problems that may be encountered in attempts to inactivate equilibrium mixtures of enzyme-substrate and enzyme-product complexes without producing quenching artifacts are considered.

An evaluation of the change in chemical potential of the covalently bound PO_3 group of phosphoglucomutase on substrate binding (see the accompanying paper, Ray and Long, 1976a) is possible if the equilibrium distribution among the enzyme-substrate and enzyme-product complexes, i.e., the central complexes, can be measured. Such a measurement can be made if a procedure is available for inactivating a mixture of central complexes sufficiently rapidly to preclude their interconversion. Because the turnover number of phosphoglucomutase is about 550 s^{-1} at room temperature (Ray et al., 1972), and because interconversion of the central complexes must be at least this fast, the inactivation must be rapid, indeed, if quenching artifacts are to be avoided. Moreover, because such a study requires the use of relatively high enzyme concentrations—so that substrate and product are saturated by enzyme (see Ray and Long, 1976a)—it is desirable to be able to quench small aliquots of equilibrium mixtures, e.g., 50 μl or less. This paper describes the construction of a simple apparatus that appears to satisfy both of the above criteria, although results in accompanying papers (Ray and Long, 1976a,b) show that some interconversion of central complexes accompanies the quenching process when the Mg^{2+} form of the enzyme is used. With less active metal forms of the enzyme, very little interconversion apparently occurs.

Experimental Section

Materials. ^{32}P-Labeled phosphoglucomutase was prepared (Long and Ray, 1973) and converted to its Mg^{2+} complex (Ray and Long, 1976a). All other materials were of reagent grade or better.

Construction and Operation of a Slow Delivery–Rapid Quench Apparatus. A motor-driven syringe control (Aminco Model A-2304A[1]) with a mechanical display showing the position of the piston was rewired so that it could be run in a variable-speed mode in both the forward and reverse directions. Adapters to hold the barrel of a 0.25-ml "Leur lok" syringe (glass) and to act as a self-centering drive for the syringe plunger also were fabricated. A 10-mm length of 3-mm i.d. Pyrex tubing served as a reservoir (0.07-ml capacity) for the solution to be quenched (see Figure 1). The reservoir was attached to the syringe by means of a Leur Kel-F adapter (Kontes, K-422373) and was tapered at the opposite end to about 1-mm o.d. The large end of a polyethylene delivery tip was drawn over the small end of the reservoir. This tip was made from polyethylene tubing of about 1-mm o.d.[2] by gently warming a narrow section over a gas micro burner, drawing it to a very small diameter, cooling to 4 °C, and cutting with a razor blade to give an overall length of about 15 mm. Tips with orifice diameters of about 35 μm and overall diameters of less than 120 μm were selected by examining with a telescopic microscope so that a clear view of the cross-section could be obtained.[3] (Even after practice, only about one tip in 40 was acceptable.) Selected tips were then cut at an angle of about 45° in the manner indicated above.

The syringe control plus reservoir and tip were mounted vertically as is shown in Figure 1. A "lab-jack" with vertical guides to reduce horizontal "play" was positioned beneath the syringe control. A high-speed magnetic stirrer with an external speed control (Tri R, Model MS 7) was attached to the top of the jack and a Plexiglas holder with a socket and set screw was fastened to the top of the stirrer so that a cylindrical quenching vessel with a flat bottom (15 mm o.d., 10 mm high) could be centered on the stirrer (see Figure 1). The quenching vessel contained 0.55 ml of quenching solution and was equipped with a Teflon-coated Alnico stirring bar that had been machined to a parallelepiped about 2 × 2 × 12 mm from a 3 × 13 mm spin bar with a circular cross section (Bel Art). A flat Teflon disc was used to close the top of the quenching vessel to limit the height of the liquid in the vessel during stirring. A small circular entry port of about 2-mm diameter was made in the

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[1] This item has been discontinued. However, any motor driven syringe that can be mounted vertically and that will perform as described could be used.

[2] Polyethylene tubing, 0.25 in. o.d., was heated over a low flame and drawn out to a diameter of about 1 mm.

[3] Cross-sections of most delivery tips were somewhat ellipsoidal, as was the orifice. Most experiments were conducted with tips whose orifice dimensions were about 25 × 45 μm.

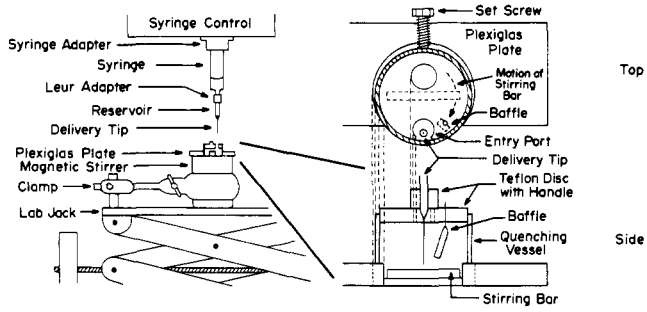

FIGURE 1: Schematic of the slow delivery-rapid quench apparatus.

disc, contiguous with the outside edge, to admit the delivery tip. A No. 27 stainless steel hypodermic needle was inserted through the disc about 2 mm "up-stream" from the center of the entry port and about 1 mm from the edge of the disc (see Figure 1). The insertion was made so that the needle slanted somewhat in the direction of the entry port. The needle was then flattened to produce a baffle, about 0.7 mm wide, which extended about 4 mm below the base of the disc (to within about 1 mm of the stirring bar). The flat side of the baffle was oriented along a ray from the center of the disc.

When correctly aligned, the quenching vessel could be rapidly raised with the lab jack to a position where the stirring bar just cleared the delivery tip. The initial horizontal positioning, which included orienting the 45° face of the delivery tip down-stream, and the final vertical positioning, during the first few seconds of the quenching process, were observed through a dissecting microscope.

The quenching procedure was initiated by displacing all air from the syringe and reservoir with diffusion pump fluid (Dow Corning 702) and slowly drawing an aliquot of about 0.05 ml of ^{32}P-labeled enzyme solution into the reservoir by operating the syringe in reverse. The syringe piston was then moved slowly forward until a positive flow was achieved, after which the position of the piston was recorded, the delivery tip wiped gently, and the quenching vessel aligned to receive the delivery tip. By raising the lab jack, the tip was rapidly passed through the entry port and pluged into the quenching solution, which was stirred at a rate of 35 rps. The stirring rate was adjusted prior to entry of the delivery tip by means of a Strobe lamp operating at the desired frequency. This lamp also allowed a much more accurate visual positioning of the delivery tip relative to the stirring bar than otherwise was possible. The syringe control was actuated manually as the tip entered the quenching mixture and after entry the delivery rate was immediately increased so that 0.04 ml was delivered in 45 ± 5 s. Because the rapid rate of stirring tended to cause cavitation of the solution, care was taken to keep the tip below the liquid surface, as close as possible to the stirring bar, and not more than 1 mm down-stream from the end of the baffle, or more than 1 mm from the wall of the quenching vessel. In fact the stirring bar frequently brushed the end of the delivery tip. When the piston reading indicated that approximately 0.04 ml had been added to the quenching mixture the flow was interrupted and the tip immediately withdrawn by lowering the lab jack. However, no attempt was made to determine the precise amount of solution actually delivered since internal standards always were used. After each use, the reservoir and attached delivery tip were rinsed with water and acetone prior to brief immersion in a sonic cleaning device: first in detergent and then in 1 N NaOH.

Label Transfer Procedure for Assessing Quenching Efficiency. Solutions of [^{32}P]phosphoglucomutase, Mg^{2+} form, were diluted in Tris chloride buffers containing 0.2 mM EDTA, 0.8 mM $MgCl_2$, and 20 mM imidazole; the final Tris concentration was 0.05–0.15 M. Aliquots were exhausted into mixtures of Glc-1-P (disodium salt), 0.2 to 0.5 M, and NaOH, 0.5 to 1.0 M, by means of the rapid quench apparatus described above. Aliquots of the quenched solution were immediately assayed for acid soluble and total radioactivity by using procedures analogous to those described previously (Long and Ray, 1973). The soluble radioactivity in runs conducted with no Glc-1-P was used as a blank value.

Results and Discussion

Even though there is no evidence that the equilibrium among the central complexes in the phosphoglucomutase reaction, E_P·Glc-1-P, E_D·Glc-P_2, and E_P·Glc-6-P,[4] is pH dependent, every reasonable effort was made to design an apparatus that would allow such a mixture to be quenched sufficiently rapidly to prelude possible interconversion of those complexes during quenching. In the initial development of this apparatus, quenching efficiency was assessed visually by exhausting an intensely colored solution of crystal violet in 0.1 M Tris into 1 N HCl and monitoring the length of the plumes of unbleached dye formed at the delivery tip by use of a dissecting microscope and a Strobe lamp (see Experimental Section). The purpose of such experiments was to minimize plume length and thus maximize the rate of quenching by producing turbulent flow, as opposed to laminar flow, in the region of the delivery tip. In fact, this procedure allowed the facile detection of many early errors in experimental design. Although pointing out the basis for the specifications outlined in the Experimental Section does not seem worthwhile, nearly all appeared to be more or less critical either to the actual quenching step or to the subsequent analysis (see Ray and Long, 1976b).

In our final apparatus the crystal violet test solution appeared to be completely bleached within a distance of 200 μm from the orifice (i.e., within a distance of less than two tip diameters). Since, under the present conditions, an exit speed of about 100 cm/s is achieved, and since complete bleaching of crystal violet in 0.1 M Tris requires mixing with 0.1 volume of 1 N HCl, this extent of mixing apparently is achieved within about 0.2 ms. (Crystal violet changes to blue color at about pH 2.8, but retains much of its color even in 0.1 N HCl; in 0.5 HCl it is a pale yellow.) In fact, the diameter of the orifice, which determines the exit rate at constant delivery time, proved to be critical in that variable results were obtained in experiments involving label transfer in the phosphoglucomutase system (see below) when delivery tips with 100-μm orifices were used under otherwise identical conditions. It is perhaps noteworthy that the radial velocity of a point on the stirring bar just beneath the delivery tip also was approximately 100 cm/s, and it is possible that the most efficient mixing is achieved when the velocity of liquids being mixed is similar.

An attempt also was made to assess quenching efficiency, in terms of the fraction of enzyme·substrate complexes that could undergo a PO_3-transfer step during the quenching process, under operating conditions. This was accomplished by (a) using ^{32}P-labeled phosphoglucomutase in the delivery

[4] The following abbreviations are used: E_P and E_D, the Mg^{2+} complexes of the phospho and dephospho forms of phosphoglucomutase, respectively; Glc-1-P, α-D-glucose 1-phosphate; Glc-6-P, D-glucose 6-phosphate; Glc-P_2, α-D-glucose 1,6-bisphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Extent of ^{32}P Transfer to Substrate on Mixing [^{32}P]-Phosphoglucomutase in Tris Buffer with Glucose 1-Phosphate–Sodium Hydroxide Solutions by Use of the Slow Delivery–Rapid Quench Apparatus.[a]

Expt	[Tris][b] (M)	[NaOH] (M)	[Glc-1-P] (M)	[Tris][c] / [NaOH]	% of Label Transferred[d]
1	0.05	1.0	0.25	0.05	6.8, 7.0
2	0.05	1.0	0.50	0.05	6.0, 6.6
3	0.05	0.5	0.25	0.15	16, 20
4	0.15	1.0	0.50	0.15	29, 30
5	0.14	0.5	0.20	0.28	67

[a] See Experimental Section for details. [b] Tris-HCl, at pH 7.5, containing [^{32}P]phosphoglucomutase at concentrations of about 0.2 mg per ml; also present were 0.8 mM Mg^{2+}, 0.2 mM EDTA, and 20 mM imidazole. [c] To a first approximation this indicates the *relative* amount of NaOH required per volume of enzyme–Tris solution to increase the pH sufficiently to produce inactivation. [d] Measured by the fraction of the total ^{32}P label found in a Cl_3CCOOH supernatant; the average of duplicate determinations is given. Double entries are from separate mixing experiments.

reservoir, (b) including substrate (Glc-1-P) in the quenching solution along with NaOH, (c) adjusting the relative concentrations of buffer in the enzyme solution and NaOH and Glc-1-P in the quenching solution so that all of the enzyme was converted to a ^{32}P-E_P·Glc-1-P complex before it was inactivated by the base in the quenching solution, and (d) measuring the extent of transfer of the labeled PO_3 group of the enzyme to bound Glc-1-P.

Making certain that all ^{32}P-E_P was converted to ^{32}P-E_P·Glc-1-P prior to inactivation was one critical aspect of this approach. However, rapid formation of this complex was facilitated by the relatively low value of the Michaelis constant, $K_{m(Glc\text{-}1\text{-}P)}$, about 8 μM, and the relatively large value of the rate constant for binding of Glc-1-P to E_P, $\geq 1.3 \times 10^8$ M s^{-1} (Ray and Peck, 1972). For example, when ^{32}P-E_P is buffered with 0.05 M Tris buffer, pH 7.5, the Glc-1-P concentration in the quenching solution is 0.5 M, and the NaOH concentration is 1 M, diluting the enzyme–Tris solution by 0.5% with the Glc-1-P–NaOH mixture causes an inappreciable pH increase (to 7.8) but produces a (Glc-1-P)/$K_{m(Glc\text{-}1\text{-}P)}$ value of about 100. Thus, more than 90% of E_P will be converted to its complex with Glc-1-P within 0.03 ms after 0.5% mixing is achieved. (In these calculations, the indicated values of $K_{m(Glc\text{-}1\text{-}P)}$ and the minimum rate constant for binding of Glc-1-P were increased and decreased, respectively, by threefold, to take into account the competitive binding of Cl^- (from the Tris buffer) and Glc-1-P (Ray et al., 1966).)

Entry 1, Table I, indicates that only 6 to 7% of the enzymic label is transferred to Glc-1-P under these conditions. Entry 2 indicates that doubling the Glc-1-P concentration does not alter the extent of transfer, as is expected if the enzyme is fully saturated with Glc-1-P prior to inactivation, and if the rate of inactivation, alone, determines the extent of transfer. Moreover, delaying inactivation by decreasing the concentration of NaOH in the quenching solution by one-half, entry 3, increases the transfer by two- to threefold. Delaying inactivation by increasing the buffering capacity of the enzyme solution by threefold, entry 4, also causes an increase in transfer (three- to fourfold). Hence, very little transfer would be expected if the sodium hydroxide concentration is increased by fourfold while the Tris concentration is decreased by 2.5-fold relative to the concentrations indicated in entry 1, i.e., if the Tris/NaOH ratio is 0.005, which is that used in an accompanying paper (Ray and Long, 1976a), instead of 0.05 as in entry 1. However, a direct measurement of transfer under the latter conditions is not possible because saturation of the enzyme with Glc-1-P prior to denaturation cannot be demonstrated under these conditions.

Similar experiments might have been conducted with E_P·Glc-6-P and with E_D·Glc-P_2 to show that inactivation of these complexes also is sufficiently rapid to allow a mixture of all three central complexes to be inactivated rapidly enough to avoid quenching artifacts. We did not attempt this partly because of technical problems, and partly because the results still would not have provided a conclusive test of quenching efficiency. Thus, many enzymic processes include an unimolecular transition subsequent to the (bimolecular) substrate binding step and prior to the catalytic step. Presumably this unimolecular step is required to produce the catalytically active enzyme·substrate complex. Although experiments of the type described above may show that *an* enzyme–substrate complex can be inactivated with the required efficiency, such experiments cannot prove that *catalytically active* enzyme·substrate complexes can be similarly inactivated. Hence, as a criterion of quenching efficiency, we have sought to obtain similar results on quenching equilibrium mixtures of catalytically active complexes by means of acids or bases, and to show that the results also are independent of the concentration of acid or base used in the quenching solution. Such a similarity is observed with the Co^{2+} form of phosphoglucomutase (Ray and Long, 1976b), which has a turnover number at room temperature of about 130 per s and thus is about 0.25 as active as the Mg^{2+} form of the enzyme (Ray et al., 1972). Similar results also are observed with three other less active metal complexes of phosphoglucomutase. However, a significant difference between the results with acidic and basic quenching solutions is obtained with the Mg^{2+} form of the enzyme. This difference apparently is unrelated to mixing problems since the results obtained with either acid or base are both reproducible and independent of the concentration of the quenching solution. Possible reasons for the difference between acidic and basic quenching steps are noted in the accompanying paper (Ray and Long, 1976a).

Since lag times of less than about 10^{-3} s cannot be obtained with most stopped-flow or rapid-flow devices, it seems reasonable to point out that complete mixing is not required to produce the desired results in the type of experiments described here, and that effective quenching times of less than 10^{-3} s are not unreasonable. Thus, mixing one volume of phosphoglucomutase in 20 mM Tris, pH 7.5, with only 0.01 volume of 2 N NaOH will raise the pH of the enzyme solution to 12. At hydrogen ion equilibrium there should be no significant reaction at pH 12 during a time interval long enough to allow denaturation of the enzyme. Moreover, the critical time interval is not even the length of time required to obtain a given extent of mixing. Rather, it is the *difference* in the time interval required to obtain sufficient mixing to produce a small but tolerable pH change and that required to produce a pH change large enough to eliminate catalysis during subsequent unfolding of the enzyme.

However, a problem will arise if all groups whose ionic state is critical to catalysis are shielded from the aqueous environment in the enzyme·substrate complex, so that dissociation of substrate is required to initiate inactivation at any pH. In fact, in systems where only the anionic forms of the substrates bind

to the enzyme, the use of an acidic quenching solution may well be more effective than a basic one, since rapidly protonating the anionic group of substrate in an enzyme·substrate complex may well be the most efficient way of eliminating further catalytic activity. In fact this expectation appears to hold in the phosphoglucomutase system (Ray and Long, 1976a).

In any case, although we feel that the apparatus described here can provide adequate rates of mixing to allow the investigation of the equilibrium ratios of central complexes present in other enzymic systems, the primary problem in such studies may not be mixing, per se, but the rate of inactivation subsequent to mixing, and each case will require a separate examination.

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Thermodynamics and Mechanism of the PO_3 Transfer Process in the Phosphoglucomutase Reaction†

W. J. Ray, Jr.,*,‡ and J. W. Long§

ABSTRACT: The equilibria among the central complexes in the phosphoglucomutase system were evaluated by (a) using an excess of enzyme plus Mg^{2+} to prepare mixtures with glucose phosphates in which essentially no *free* glucose phosphates were present; (b) inactivating the enzyme in such mixtures by means of a procedure that prevents substantial interconversion of the central complexes; and (c) assaying the quenched mixture for glucose-1-P, glucose-1,6-P_2, and glucose-6-P. The fractional amounts of E_P·Mg·Glc-1-P, E_D·Mg·Glc-P_2, and E_P·Mg·Glc-6-P present at pH 7.5 and 24 ° C were 0.13, 0.54, and 0.33. (E_P and E_D are the phospho and dephospho forms of the enzyme, respectively.) From these fractions and the equilibrium isotope exchange constants for the three sugar phosphates, true dissociation constants can be calculated for each of the above complexes: 8.5 μM, 19 nM, and 57 μM, respectively. Relative to the rate of PO_3 transfer to water, a 3×10^{10}-fold rate increase is produced by binding glucose-1-P to the Mg^{2+}-enzyme (Ray, Jr., W. J., Long, J. W., and Owens, J. D. (1976), *Biochemistry,* the following paper in this issue.) This "substrate-induced rate effect" is equivalent to a difference of some 14 kcal in Gibbs activation energies for transfer to chemically similar hydroxyl groups, and most of this energy difference ultimately must be rationalized in terms of binding interactions involving the phosphoglucosyl moiety. Three different mechanisms for using substrate binding energy to reduce the activation energy of the subsequent catalytic step are examined as possible explanations for the substrate-induced rate effect. These mechanisms emphasize (a) enthalpic destabilization and (b) (entropic) immobilization of *reactant groups* during *formation* of the *enzyme–substrate complex,* and (c) increased binding interactions of *nonreactant groups* during the subsequent *approach to* the *transition state.* As a test for enthalpic destabilization of the enzymic phosphate group, values of $\Delta G^{\circ\prime}$ for the hydrolytic cleavage of this group in E_P and E_P·Glc-1-P are calculated from equilibria measured at pH 7.5 and 30 °C: about −1 and +1.4 kcal/mol, respectively. To test for destabilization of the acceptor hydroxyl group in the enzyme–substrate complex, $\Delta G^{\circ\prime}$ for the equilibrium, E_P·Glc-P $\rightleftharpoons$ E_D·Glc-P_2, is compared with that for the corresponding process involving the nonrigid acceptor, 1,4-butanediol monophosphate: about −0.9 and −1.9 kcal, respectively. These results are not consistent with a large enthalpic destabilization of the reactant groups in the E_P·Glc-1-P complex. To test for entropic immobilization of reactant groups, glucose 6-phosphate is considered as a bidentate ligand, and the chelate effect on the binding and subsequent enzymic transfer reaction that arises from covalently linking the sugar ring and the PO_3 group is evaluated. Reference reactions involving xylose as a PO_3 acceptor both in the presence and absence of bond (inorganic) phosphite are used. The covalently attached $CH_2OPO_3^{2-}$ group of glucose-6-P contributes about −6.2 kcal/mol to $\Delta G^{\circ\prime}$ for binding and reduces $\Delta G^{\ddagger}$ by about 7.2 kcal/mol, while $\Delta G^{\circ\prime}$ for binding of phosphite (in the presence of bound xylose) is about −5.6 kcal/mol (calculated by use of mole-fraction binding constants) and its binding reduces $\Delta G^{\ddagger}$ by about 6.2 kcal/mol. Since the overall effect (sum of $\Delta G^{\circ\prime}$ and $\Delta G^{\ddagger}$) is only about 1.6 kcal/mol more negative for the *covalently attached* $CH_2OPO_3^{2-}$ group than the *independent* HPO_3^{2-} molecule, the chelate effect for glucose-6-P is marginal, the bidentate interaction of the component parts of glucose-6-P with the enzyme does not appear to involve an unusual degree of immobilization, and the rate effect produced by immobilization of reactant groups does not approach its theoretical limit; i.e., it does not contribute overwhelmingly to the substrate-induced rate effect. To test whether binding interactions involving *nonreactant groups* markedly increase in proceeding from the enzyme–substrate complex to the transition state, an attempt was made to circumvent a possible mechanism for *preventing* maximal binding interactions between the enzyme and such groups in the enzyme–substrate complex: using the 6-hydroxymethyl group of the reactant to

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