Okunuki, K., & Sekuzu, I. (1954) *Seitai no Kagaku 5*, 265-272.
Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375-380.
Robinson, N. C., Neumann, J., & Wiginton, D. (1985) *Biochemistry 24*, 6298-6304.
Rosevear, P., VanAken, T., Baxter, J., & Ferguson-Miller, S. (1980) *Biochemistry 19*, 4108-4115.
Sinjorgo, K. M. C., Meijling, J. H., & Muijsers, A. O. (1984) *Biochim. Biophys. Acta 767*, 48-56.
Thompson, D. A., & Ferguson-Miller, S. (1983) *Biochemistry 22*, 3178-3187.
Tonomura, B., Nakatani, H., Ohnishi, M., Yamaguchi-Ito, J., & Hiromi, K. (1978) *Anal. Biochem. 84*, 370-383.
van Buuren, K. J. H., Zuurendonk, P. F., van Gelder, B. F., & Muijsers, A. O. (1972) *Biochim. Biophys. Acta 256*, 243-257.
van Gelder, B. F. (1978) *Methods Enzymol. 53*, 125-128.
Vik, S., & Capaldi, R. A (1980) *Biochem. Biophys. Res. Commun. 94*, 348-353.
Yonetani, T. (1960) *J. Biol. Chem. 235*, 845-852.

Observation of a Kinetic Slow Transition in Monomeric Glucokinase†

Kenneth E. Neet,* Robert P. Keenan, and Peter S. Tippett
Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106
Received May 30, 1989; Revised Manuscript Received August 31, 1989

ABSTRACT: Rat liver glucokinase (EC 2.7.1.2) is a monomeric enzyme with positive cooperativity for glucose phosphorylation for which several kinetic mechanisms have been proposed. We have observed a slow kinetic transition when the enzyme is assayed in the presence of 30% glycerol. When the enzyme had been preincubated or stored in 50 mM glucose, the initially rapid activity decayed, via a first-order process, to a new steady-state velocity. The glucose-induced process is reversible since if the enzyme is preincubated without glucose, an initially low activity accelerates over minutes to the same steady-state velocity. This final velocity is independent of the preincubation conditions and is determined solely by the glucose and ATP concentrations in the assay. Possible artifacts which might cause nonlinear progress curves have been ruled out. The transition has a half-time of 2-10 min depending on glucose and ATP concentrations and temperature. In the steady-state kinetics, positive cooperativity occurs with glucose with a Hill coefficient (n_H) = 1.3 at high ATP concentrations, approaching unity as the ATP concentration decreases. This pattern is similar to that seen in the linear velocities in the absence of glycerol. Similarly, negative cooperativity with MgATP is seen in the steady-state velocities at low glucose concentrations with the Hill coefficient approaching 1 as the glucose concentrations approach saturation. The initial velocity for enzyme preincubated in high glucose concentration was either Michaelis-Menten as a function of glucose at high MgATP concentration or heterogeneous ($n_H < 1$, negatively cooperative) at low MgATP concentration. Preincubation with the competitive inhibitor *N*-acetylglucosamine eliminated the cooperativity in the absence or in the presence of glycerol and also eliminated the slow transition, presumably by preferentially binding to one of the active conformations of glucokinase. Other monosaccharide substrates had individualized transition times and cooperativity patterns. We propose that this hysteretic behavior observable in glycerol is due to a conformational change and is the molecular basis for the kinetic cooperativity with the monomeric enzyme, glucokinase, under normal assay conditions. Our data require two catalytic cycles and thus support the slow transition model [Neet, K. E., & Ainslie, G. R., Jr. (1980) *Methods Enzymol. 64*, 192-226; Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1984) *Eur. J. Biochem. 145*, 163-171] that is consistent with the known kinetic and physical properties of glucokinase.

Glucokinase (EC 2.7.1.2) is a hepatic enzyme which participates in the short-term regulation of blood glucose levels. Evidence from several laboratories (Storer & Cornish-Bowden, 1977; Cardenas et al., 1979) has shown that glucokinase kinetics are positively cooperative with glucose. Functionally, this cooperativity (Hill coefficient = 1.5) probably allows glucokinase activity to have an increased sensitivity to fluctuating blood glucose levels in the physiological range (Bontemps et al., 1978). Mechanistically, however, it is difficult to explain this cooperativity in classical terms, since several studies have shown glucokinase to be a 50000-dalton monomer (Holroyde et al., 1976; Cardenas et al., 1978) with only a single glucose binding site and no evidence of oligomerization.

Ligand-induced slow transitions (Ainslie et al., 1972) and mnemonic mechanisms (Ricard et al., 1974) have been utilized to explain possible kinetic cooperativity in monomeric enzymes. Hysteretic enzymes (Frieden, 1970) that display a slow transient during assay have been related to this kinetic cooperativity through the mechanism of Ainslie et al. (1972). The basic assumptions of either a mnemonic or a ligand-induced slow transition mechanism in a monomeric enzyme are the existence of at least two kinetically distinct conformational

† This investigation was supported in part by National Institutes of Health Grant AM12881 and by the Diabetes Association of Greater Cleveland. A portion of this work is taken from a thesis submitted by P.S.T. to Case Western Reserve University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
* To whom correspondence should be addressed.

0006-2960/90/0429-770$02.50/0

forms of the enzyme and the ability for these forms to interconvert through a relatively slow conformational change. The distinction between the mnemonic mechanism and the general slow transition mechanism lies in the assumption of the mnemonic mechanism that only one form of the enzyme, the free enzyme, may assume a second conformation; i.e., there is only one active enzyme–substrate complex or catalytic cycle (Neet & Ainslie, 1980; Cornish-Bowden & Cardenas, 1987). Additionally, in order to generate cooperativity by either mechanism, the relative populations of each form must be dependent upon the concentration of the cooperative substrate (Neet & Ainslie, 1980). Storer and Cornish-Bowden (1977) applied a slightly modified form of the mnemonic mechanism (Ricard et al., 1974) to the glucokinase steady-state kinetics and subsequently supported the mechanism with isotope exchange studies at equilibrium (Gregoriou et al., 1981) and solvent isotope effects (Pollard-Knight et al., 1985). These studies also showed that glucokinase follows a predominantly ordered mechanism with glucose binding first and glucose 6-phosphate released last. Cardenas et al. (1979) proposed a similar mnemonic mechanism to explain the cooperative glucose kinetics in glucokinase, but then extended it to encompass the more general form of the slow transition mechanism (Olavarria et al., 1982; Cardenas et al., 1984a) based on several new steady-state observations. A recent review concurs that the slow transition mechanism better describes the substrate analogue data (Cornish-Bowden & Cardenas, 1987).

A hysteretic progress curve due to a slow change in the distribution of active species of glucokinase has not previously been observed nor has any direct evidence for the existence of more than one catalytic isoform of glucokinase been reported. The current work reports that in the presence of glycerol at least two slowly interconvertible, kinetically distinct forms of glucokinase are demonstrable. The consequent hysteresis in the progress curve represents a temporal shift in the relative populations of these forms. The true steady-state velocities with and without glycerol have similar properties. These data are interpreted in terms of the slow transition mechanisms.

Materials and Methods

Glucokinase was prepared by modification (Tippett, 1981) of the method of Holroyde et al. (1976). Like the work of Storer and Cornish-Bowden (1976, 1977), most of this work was with enzyme purified through the affinity column stage that had a specific activity more than 80 units/mg. Kinetic comparisons between enzyme from this stage and homogeneous enzyme (150 units/mg) have always given identical results here, as elsewhere (Storer & Cornish-Bowden, 1976, 1977). Pyruvate kinase/lactate dehydrogenase was from Boehringer-Manheim. All other compounds were the best grade available and were obtained from either Sigma or Fisher.

Unless otherwise indicated, glucokinase was assayed in an assay mixture containing 30% (v/v) glycerol, 1 or 2 mM dithiothreiol (DTT),[1] 2–5 units of glucose-6-phosphate dehydrogenase (Leuconostoc), 0.5 mM NAD, 100 mM triethanolamine, pH 7.5, 5 mM MgATP, 5 mM excess (over MgATP) $MgCl_2$, and variable concentrations of freshly prepared β-D-glucose. In some experiments, 1 mM phosphoenolpyruvate and 5 units of pyruvate kinase were included such that both products were then removed by subsequent reactions. In these latter experiments, the ATP concentration remained constant, and the glucose concentration never changed by more than 5–10% over the entire course of the assay. In separate experiments, the glucose 6-phosphate formed was coupled through phosphoenolpyruvate (1 mM), NADH (0.5 mM), and pyruvate kinase/lactate dehydrogenase (2–10 units). In various experiments, individual components were varied, including glycylglycine (100 mM, pH 8.5) as the buffer, 0.05–5 mM MgATP, 0–5 mM excess $MgCl_2$, 0–150 mM KCl, glycerol, or coupling enzymes. Unless otherwise noted, the reaction was initiated with glucokinase (80–100 units/mg) that had been stored in 20 mM triethanolamine, pH 7.0, 5 mM EDTA, 2 mM DTT, 50 mM glucose, 400 mM KCl, and 30% glycerol (high glycerol preincubation) or dialyzed overnight against the same buffer without glucose (low glucose preincubation).

Reactions were followed by the change in the absorbance at 340 nm for approximately 20–30 min, and data were collected on a Gilford spectrophotometer, Model 252, updated with the System 2600 microprocessor and recorded on diskettes with a NorthStar Horizon microcomputer. This arrangement allowed the acquisition of absorbance data, accurate to 0.001 unit, every 8 s (or longer intervals) and storage on floppy diskettes. Subsequent mathematical analysis of the data utilized a program written in Basic (Furman & Neet, 1982) that performs a nonlinear least-squares fit with the Marquardt algorithm (Bevington, 1969) to any appropriate equation. The equation used for these fits [eq 1 of Neet and Ainslie (1980)] was for a single-exponential decay of one enzyme form to another and yielded the fitted values for the initial velocity (v_i), the steady-state velocity (v_{ss}), and the transition time (τ). In order to facilitate the analysis, some data sets were initially fit to linear steady-state velocities at 15–20 min and then fit to the v_i and τ. For each experiment, the individual data points were automatically plotted on a Hewlett-Packard 7225A X–Y plotter interfaced to the Horizon microcomputer, along with a curve simulated to the values of the fitted parameters. Analysis of selected curves by semilog plots (Neet & Ainslie, 1980) and the goodness of fit by the computer analysis demonstrated that the transient followed first-order kinetics and that this algorithm for obtaining kinetic parameters was optimal. For measurement of Hill coefficients (v_i or v_{ss}), assays were done in duplicate or triplicate either with 6–12 glucose concentrations ranging from 0.25 to 100 mM or with 12 concentrations of MgATP ranging from 0.05 to 5 mM. Since the transient was not visible above about 10 mM glucose, the steady-state and initial velocities were taken to be identical above that point. Determination of kinetic parameters for the appropriate velocity was done either (a) by graphical estimation of the maximal velocity from a double-reciprocal plot and calculation of the Hill coefficient (n_H) and half-saturation ($S_{0.5}$) from a Hill Plot or (b) by simultaneous determination of all three parameters from the Hill equation by the fitting program (unweighted) described above. Similar values were obtained by either procedure, but when sufficient data points were available (>8), the latter was preferred since it is intrinsically less biased.

Results

Observation and Validation of the Assay Transient. Glucokinase is normally (Holroyde et al., 1976) stored in a buffer containing 30% glycerol and high concentrations (50 mM) of glucose (Holyrode et al., 1976; Tippett, 1981). When glucokinase stored in this way was assayed (upper curve, Figure

[1] Abbreviations: DTT, dithiothreitol; G6PD, glucose-6-phosphate dehydrogenase; GlcNAc, *N*-acetylglucosamine; PEP, phosphoenolpyruvate; PK/LDH, pyruvate kinase–lactate dehydrogenase; K_m, Michaelis constant; K_i, inhibition constant; n_H, Hill coefficient; $S_{0.5}$, substrate concentration for half-maximal activity.

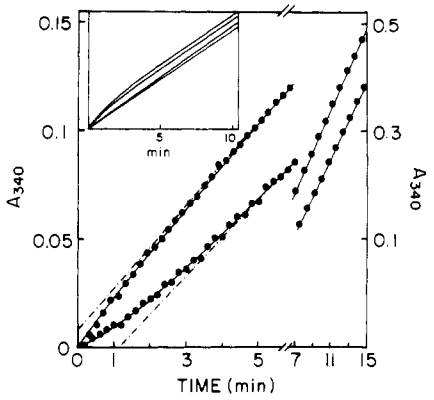

FIGURE 1: Typical progress curves in glycerol. Glucokinase was stored in a buffer containing 50 mM glucose (burst, upper curve) or dialyzed to remove glucose before assay (lag, lower curve). Final assay conditions were identical in both cases: 100 mM TEA, pH 7.5, 2 mM DTT, 0.5 mM NAD, 2 units of G6PDH, 0.25 mM ATP, 10 mM $MgCl_2$, 1.0 mM glucose, and 30% glycerol (w/v) at 30 °C. For these experiments, the reaction was initiated by the addition of glucose. The steady-state velocity was linear up to at least 15 min. Points were digitized every 8 s, but alternate points are omitted for clarity. Solid lines are fitted curves to an exponential process with the following parameters: stored in glucose, $v_i = (2.44 \pm 0.09) \times 10^{-2}$ AU/min, $v_{ss} = (1.91 \pm 0.005) \times 10^{-2}$ AU/min, $\tau = 1.38 \pm 0.24$ min; stored without glucose, $v_i = (0.68 \pm 0.12) \times 10^{-2}$ AU/min, $v_{ss} = (1.77 \pm 0.01) \times 10^{-2}$ AU/min, $\tau = 2.07 \pm 0.16$ min. Inset: Progress curves for glucokinase that had been stored in 50 mM glucose and assayed at concentrations of 0.25, 0.5, 1, or 5 mM glucose (top to bottom curves, respectively) and 5 mM MgATP.

1) in an assay mixture also containing 30% glycerol, the rate was rapid initially but decreased to a slower steady-state velocity (burst) over a period of minutes, after which the progress curve was linear. The extent and the time course of the "burst" in the progress curve were dependent on the glucose concentration (inset, Figure 1). The amount of product formed in the burst over a period of minutes clearly indicates that the observed process represents multiple turnovers of the enzyme and not a stoichiometric event related to the enzyme concentration. The same enzyme assayed in the normal assay in the absence of glycerol showed no observable kinetic transition from pH 6 to 9 (data not shown). Since the enzyme was being transferred from high glucose (storage) to low glucose concentration (assay of Figure 1), we reasoned that removal of the glucose from the enzyme before assay would result in a progress curve that increased in activity during the assay period, i.e., a lag. The result of this experiment is shown by the lower curve in Figure 1. The linear portion of the curve had identical rates regardless of the preincubation conditions, as would be expected for a thermodynamically controlled, true steady state. The observed lag had approximately the same half-time for attainment of the steady state. Furthermore, the kinetic constants for the steady state had similar values for the glucose parameters regardless of the initial condition: 1.50 (±0.15) mM vs 1.97 (±0.13) mM for $S_{0.5}$; 1.53 (±0.16) vs 1.36 (±0.09) for the Hill coefficient. The lag was not analyzed for v_i because of the difficulty of reproducibly removing glucose and the relative instability of the enzyme under these conditions.

Several possibilities were considered in order to rule out the possible presence of an artifact or other trivial explanation as the cause of these nonlinear progress curves, as distinguished from a true ligand-dependent conformational change in the enzyme. (a) The burst was not due to either the depletion of substrates or the accumulation of products, since its properties were invariant whether or not all products were removed by subsequent reactions. Values of v_i, v_{ss}, and τ were within experimental error whether coupling was through glucose-6-phosphate dehydrogenase or through ATP via pyruvate kinase and lactate dehydrogenase or whether glucose 6-phosphate was removed and ATP recycled by the simultaneous presence of glucose-6-phosphate dehydrogenase, phosphoenolpyruvate, and pyruvate kinase (see Materials and Methods). Some reactions were carried out in which less than 1% of the glucose and none of the ATP (since it was recycled) were consumed and the character of the burst was unchanged. Increasing the coupling enzyme concentration above the levels indicated had no effect. (b) The burst was not due to a surface denaturation phenomemon since no effect was observed if albumin (1 mg/mL) were included in the assay or if the assay were carried out in plastic rather than glass cuvettes. (c) The effect was also not due to contamination of ATP with Al^{3+} that has been reported to affect the kinetics of yeast hexokinase (Womack & Colowick, 1979; Viola et al., 1980; Neet et al., 1982). Addition of Al^{3+} or citrate had minimal effect on the rate and no effect on the transient. Furthermore, the presence of AlATP would not account for positive cooperativity with the other substrate, glucose. (d) The transition observed here is different from the oxidative inactivation through sulfhydryl groups previously described (Tippett & Neet, 1983). Reducing agents do not prevent or reverse the transient in glycerol discussed here. Nevertheless, DTT was included in these assays so that no oxidation could occur. Furthermore, the 30% glycerol used here to make the hysteresis observable strongly protects the enzyme from sulfhydryl oxidation and inactivation (Tippett & Neet, 1983). (e) Glucokinase has been reported to show a 2-fold lower half-saturation and a 15–20% lower maximal velocity with the α-D anomer of glucose (Meglasson & Matschinsky, 1983) and thus might demonstrate an apparent transient as one form was used up in the assay. No difference in the transient behavior was observed between freshly made solutions of β-D-glucose (Sigma) and either the same solution after aging to allow equilibration between the anomers or solutions of "dextrose" (Fisher Chemical) containing an equilibrium mixture of anomers. Moreover, the amounts of glucose utilized in the first 10 min of assay are not sufficient to consume one anomer. Further evidence against this possibility is the observation of the transient in glycerol with several other sugar substrates (see below).

The only essential component for visualization of the transient was the glycerol. Indeed, a gradation of the effect was observable at intermediate glycerol concentrations. For example, in 5% glycerol, assays were essentially linear with kinetic constants indistinguishable from those without glycerol. The clearly demonstrable reversal of the transition by removal of glucose in the preincubation (Figure 1) also strongly argues against a trivial explanation of the transient and led to the postulate that the primary effect of glycerol in the assay mixture is to significantly increase the half-time of a normal conformational change in glucokinase mediated by glucose during catalysis. Other properties of the transient were consistent with this interpretation. (a) The transition time (τ) was independent of glucokinase concentration, within experimental error, over the 20-fold range tested from 0.03 to 0.7 unit/mL (equivalent to 0.2–5 μg/mL) (data not shown). Even though cells with different light paths were utilized for these experiments, the data were difficult to quantitate accurately. These results suggest that an enzyme association/dissociation was not the cause of the nonlinear progress curves and are consistent with the lack of any reported oligomeric structure for glucokinase. (b) The transient was observable at all stages of purity of glucokinase, from specific activities of 10 to over

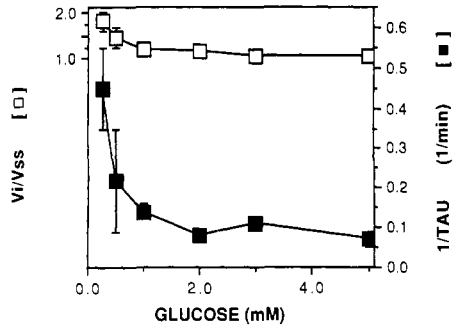

FIGURE 2: Reciprocal relaxation time, τ^{-1} (■), and velocity ratio, v_i/v_{ss} (□), as a function of glucose concentration in 30% glycerol. Assay conditions were as in Figure 1 with 5 mM MgATP. The glucokinase had been stored in 50 mM glucose. The ratio of the initial to the steady-state velocities and the relaxation times were determined from computer fits to an exponential equation as described under Materials and Methods. Values shown are mean ± the range of replicates. Relaxation times above 5 mM glucose could not be determined because of the near-equality of the initial and steady-state velocities under these conditions.

100 units/mg. (c) The essential properties of the burst were not changed by alternate buffer conditions. Assays performed in glycylglycine from pH 8.0 or 8.5 or in triethanolamine from pH 7 to 8.5 all showed the presence of the transient with only slightly altered parameters. The presence or absence of 100 mM KCl, a known stabilizer of glucokinase, did not eliminate the burst. (d) Our standard assay uses Mg^{2+} in 5 m excess over the molar concentration of ATP. This concentration causes a slight inhibition of glucokinase activity but maintains maximal amounts of complexed MgATP. No effect on the transient was observed from Mg^{2+} equimolar to ATP to a 10 mM excess of Mg^{2+} over ATP (0.25 mM), thus indicating that this ion neither caused nor contributed significantly to the transition of the enzyme (data not shown). (e) Temperature studies showed that the τ value increased by about 5-fold when the temperature was decreased from 30 to 10 °C. (f) The extent and rate of the burst were dependent on glucose and on MgATP concentrations. The value for τ^{-1} for the first-order transition showed a statistically significant decrease with increasing glucose concentration (Figure 2). In addition, the extent of the transition, as measured by the ratio between the initial and steady-state velocities, was dependent upon glucose concentration. The burst was greatest at low glucose concentrations and was nearly nonobservable ($v_i = v_{ss}$) at glucose concentrations above approximately 5 mM. This convergence of velocities accounts for the difficulty in obtaining good relaxation data at high glucose concentrations. The transient was most obvious at lower MgATP concentrations, but the τ data were not analyzed as a function of MgATP concentration because of the difficulty of obtaining reliable values.

Kinetic Properties with Glucose in Glycerol. The kinetics of the initial and steady-state velocities were examined in the 30% glycerol assay system to determine whether the kinetics resemble the published data (obtained without glycerol) and to further define this hysteresis and its relation to the kinetic mechanism of glucokinase. Glucokinase was assayed at pH 7.5 in an assay mixture containing 30% glycerol, 6–24 glucose concentrations from 0.25 to 100 mM, and either high (5 mM) or low (0.25 mM) concentrations of MgATP. These progress curves were followed over 30 min and analyzed as described under Materials and Methods to obtain values for the initial and steady-state velocities. The velocity data were then analyzed by the fitting program (see Materials and Methods) to obtain n_H, V_{max}, and $S_{0.5}$ and replotted on appropriate double-reciprocal and Hill plots for visual inspection. Results

Table I: Kinetic Constants for Glucose with Glucokinase in Glycerol[a]

conditions	Hill coefficient[b]	$S_{0.5}$[b] (mM)	V_{max}[b] (relative)
0% glycerol			
5.0 mM ATP	1.63 ± 0.09 (4)	4.8 ± 0.8 (4)	1.0
0.05 mM ATP	1.1 ± 0.12 (2)	3.5 ± 0.3 (2)	0.1
30% glycerol[c]			
5.0 mM ATP, v_i	1.11 ± 0.06 (3)[d]	1.7 ± 0.3 (3)	0.6
5.0 mM ATP, v_{ss}	1.3 ± 0.12 (5)[d]	2.8 ± 0.7 (5)	0.6
0.05 mM ATP, v_i	0.65 ± 0.15 (2)[d]	0.8 ± 0.04 (1)	0.15
0.05 mM ATP, v_{ss}	1.08 ± 0.08 (2)[d]	1.0 ± 0.1 (1)	0.15

[a] Glucokinase stored in 50 mM glucose was assayed as described in Figure 1 at 5.0 mM MgATP or 0.05 mM MgATP, with 5 mM excess Mg^{2+}, either in the absence or in the presence of 30% glycerol. [b] Determined from a nonlinear least-squares fit to initial (v_i) or steady-state (v_{ss}) velocities measured in duplicate at 6–12 glucose concentrations from 0.1 to 100 mM. Values given as the weighted mean of the parameters from two to five independent experiments plus or minus the standard deviation (number of experiments). Where only one experiment is indicated, the standard error of the fit is shown. [c] Velocities determined from a nonlinear least-squares fit to the nonlinear progress curve for a single-exponential decay at each concentration. [d] Analysis of statistical significance by the Student's t test: 1.3 is different from 1.6 ($p < 0.01$) and different from unity ($p < 0.01$); 0.65 is different from unity ($p < 0.1$); 1.11 and 1.08 are not significantly different from unity ($p > 0.1$).

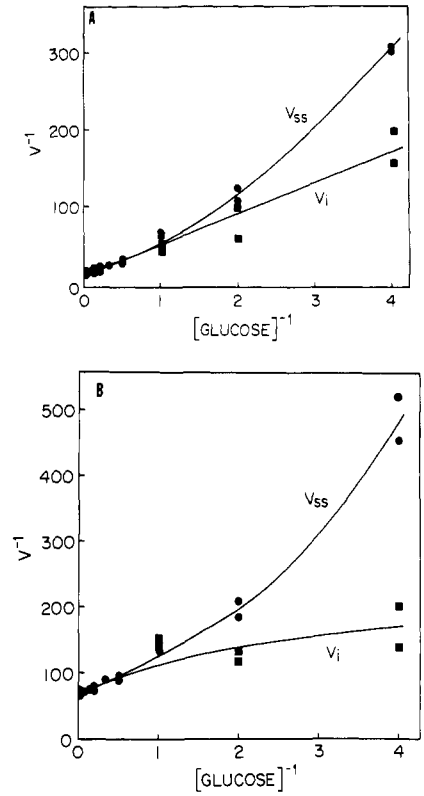

FIGURE 3: Glucose double-reciprocal plot in 30% glycerol. The v_i (■) and v_{ss} (●) for glucokinase were determined by a computer fit at varying concentrations (mM) of glucose. The fits shown were significantly better than the simpler assumption of no cooperativity. Velocity units are in AU/min. Assay conditions were as in Figure 1, and the glucokinase had been stored in 50 mM glucose. (Panel A) 5 mM MgATP. The fitted n_H values from this experiment for the v_{ss} and the v_i are 1.3 ± 0.06 and 1.1 ± 0.078 respectively. (Panel B) 0.05 mM MgATP. The fitted n_H values in the experiment for the v_{ss} and the v_i are 1.16 ± 0.07 and 0.58 ± 0.10, respectively.

are summarized in Table I, and representative data are shown in Figure 3. Several important generalizations can be made from these results.

In the steady-state velocities, the enzyme showed some of the same properties as when assayed without glycerol. Positive

Table II: Inhibition of Glucokinase by *N*-Acetylglucosamine (GlcNAc)[a]

assay conditions	preincubation addition[c]	Hill coefficient[b]	V_{max}[b] (relative)	V_{max}[b] (relative)
0% glycerol				
GlcNAc, 10 mM	none	1.35	250	0.96
GlcNAc, 10 mM	GlcNAc	1.15	310	0.98
30% glycerol				
GlcNAc, 10 mM	none	1.5	98	0.20
GlcNAc, 10 mM	GlcNAc	1.05	160	0.27

[a] Glucokinase stored in 50 mM glucose was assayed as described in Figure 1 at 5.0 mM MgATP with 5 mM excess Mg^{2+}, in the presence of a final concentration of 10 mM GlcNAc and either in the absence or in the presence of 30% glycerol. [b] Determined from a nonlinear least-squares fit to the linear steady-state velocity measured in duplicate at 10 or more glucose concentrations from 10 to 675 mM. V_{max} is relative to the uninhibited assay without glycerol. [c] Glucokinase was dialyzed to remove glucose and preincubated with 100 mM GlcNAc for 30 min, and the reaction was initiated with glucose. When GlcNAc was not in the preincubation, the reaction was initiated with glucokinase in 50 mM glucose.

cooperativity ($n_H > 1$) was observed with glucose at high MgATP concentrations and approached no cooperativity ($n_H \sim 1.1$) at low (0.05 mM) MgATP concentrations (Table I). The $S_{0.5}$ for glucose was slightly lower ($\sim$3 mM), but the V_{max} was 45% of that measured in assays without glycerol.

In contrast to the behavior of the steady-state velocities either with or without glycerol, the initial velocities in the 30% glycerol system showed normal glucose kinetics ($n_H \sim 1.0$) at high concentrations of MgATP (Table I). The apparent $S_{0.5}$ for glucose was lowered to about 1.5 mM, and the relative V_{max} was still about 45% relative to the absence of glycerol. This effect of glycerol on kinetic constants has been reported for observed linear velocities, presumably "v_i" (Pollard-Knight et al., 1985). At low MgATP concentrations, however, the n_H for glucose was 0.6 (Figure 3B, Table I). One reason for an n_H less than 1 is if two enzyme forms with different K_m values catalyze a reaction. For glucokinase, the forms would have to preexist at the extrapolated time zero for the initial velocity and also have different $S_{0.5}$ values for MgATP. The Michaelis–Menten behavior at saturating MgATP concentrations would indicate a single kinetic form, and the Hill coefficient for glucose less than unity at low MgATP concentrations would be consistent with a kinetically heterogeneous population. Observation of $n_H < 1$ for glucose has also been made[2] in dilute buffers (Cardenas et al., 1979; Olavarria et al., 1982).

Inhibition by GlcNAc. GlcNAc is an effective competitive inhibitor with respect to glucose that has the interesting property of reducing the Hill coefficient for glucose to unity at 10 mM GlcNAc (Cardenas et al., 1979, 1984a). This behavior has been explained on the basis that GlcNAc binds preferentially to one enzyme form and prevents the conformational changes necessary for kinetic cooperativity. We reasoned that GlcNAc should also affect the slow transition observed in glycerol if the two processes were related. Indeed, assays in 30% glycerol in which dialyzed glucokinase was preincubated for 30 min with 10 mM GlcNAc had linear progress curves under the conditions in which a lag occurs without GlcNAc. With a shorter preincubation time with GlcNAc, a burst could be observed when the assay was initiated with glucose. The cooperativity and apparent inhibition constants also depended on whether or not the glucokinase was preincubated with GlcNAc (Table II). In all cases, GlcNAc was competitive with glucose; i.e., the V_{max} values are not

[2] M. L. Cardenas, personal communication.

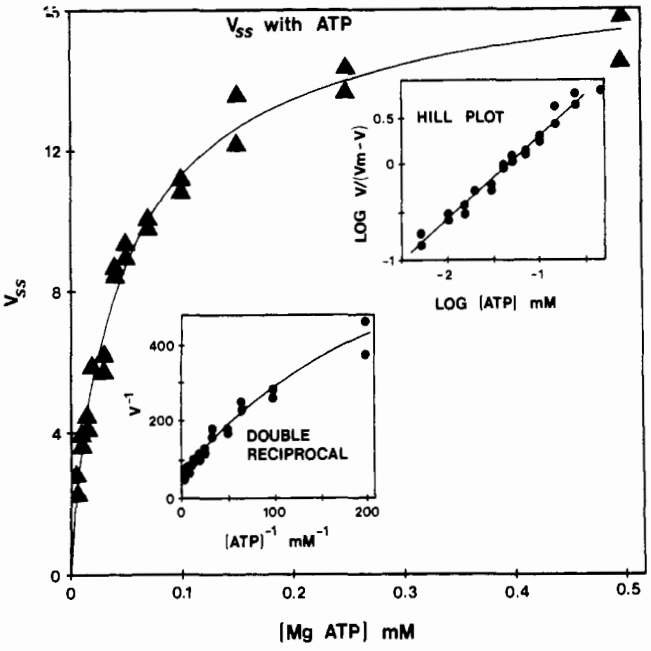

FIGURE 4: Kinetics with MgATP in 30% glycerol at low glucose concentration (0.5 mM). The v_{ss} (▲) for glucokinase was determined by a computer fit at varying concentrations (mM) of MgATP. Velocity units are in AU/min. Assay conditions were as in Figure 1. The glucokinase had been stored in 50 mM glucose. The value for n_H for the fit shown is 0.84 ± 0.07. Upper inset: Hill plot of the same data. The value for n_H from the slope of the line is 0.86 ± 0.03. Lower inset: double-reciprocal plot of the same data.

changed relative to the absence of GlcNAc (cf. Table I). The cooperativity was lower and the $S_{0.5}$(app) for glucose was higher when GlcNAc was present in the preincubation, indicating that time was required for the inhibitor to have its full effect. The ability of preincubation with GlcNAc to effect both the slow transition and the positive cooperativity in glycerol is compatible with a relation between these two phenomena. However, since this system is clearly nonequilibrium, more extensive analysis or interpretation was not done.

MgATP Kinetics. The MgATP kinetics with glucokinase in 30% glycerol for the steady-state velocities were normal hyperbolic at high glucose concentrations ($n_H = 1.0$), in concordance with controls under normal assay conditions in the absence of glycerol. However, in 30% glycerol, a distinct negative cooperativity occurred in the steady-state velocity and was readily observable at glucose concentrations below 2 mM (Figure 4). This characteristic was evident in the double-reciprocal plot (inset), and the calculated Hill coefficient was 0.84 in this case (inset). At higher glucose concentrations, the cooperativity with MgATP is lost. This substrate-dependent effect is the converse of that seen with glucose as the variable substrate. The low Hill coefficient in the steady state must be due to kinetic cooperativity since the initial velocities yielded a Hill coefficient of unity, indicating that initially a single kinetic form (with respect to MgATP) was present. The $S_{0.5}$ for MgATP in the 30% glycerol mixture at low glucose concentration was 0.13 mM. For the extrapolated initial velocities, the MgATP kinetics were Michaelis–Menten at all concentrations of glucose ($n_H \sim 1.0$), with a K_m for MgATP at saturating glucose concentration of about 0.18 mM. These results provide supporting evidence for two (or more) catalytic cycles in the glucokinase mechanism.

Alternate Substrates. Cardenas et al. (1984a,b) have made a convincing argument that glucokinase is not entirely specific for sugar substrates (and should be named as a hexokinase). We have examined other monosaccharides in the glycerol

Table III: Kinetic Constants for Alternate Substrates with Glucokinase with and without Glycerol

substrate	Hill coefficient[b]	$S_{0.5}$[b] (mM)	V_{max}[b] (relative)	τ (min)[c,d]
no glycerol				
mannose, linear	1.35	7.4	0.95	
2-deoxyglucose[d]				
v_{ss}	1.16	30	0.8	0.5–2.5
fructose[d]				
v_i	1.09	310	2.3	0.5–2.0
v_{ss}	1.44	240	2.0	
30% glycerol				
mannose[d]				
v_i	1.01	2.6	0.6	0.5–8.5
v_{ss}	1.30	3.4	0.6	
2-deoxyglucose[d]				
v_i	1.06	7.7	0.6	2.0–7.5
v_{ss}	1.19	8.5	0.6	
fructose[d]				
v_i	1.21	50	0.9	2.5–8.0
v_{ss}	1.37	51	0.9	

[a] Glucokinase stored in 50 mM glucose was assayed as described in Figure 1 at 5.0 mM MgATP or 0.05 mM MgATP, with 5 mM excess Mg^{2+}, either in the absence or in the presence of 30% glycerol. The reaction product ADP was coupled to PEP, pyruvate kinase, and lactate dehydrogenase for all substrates. Glucokinase was dialyzed against storage buffer without glucose just prior to assay such that insufficient glucose was added with the enzyme solution to affect the observed rates. [b] Determined from a nonlinear least-squares fit to initial (v_i) or steady-state (v_{ss}) velocities measured in duplicate at 10 or more monosaccharide concentrations from about 10-fold below to about 10-fold above the $S_{0.5}$. [c] τ values represent the range observed at low saccharide concentrations. [d] Velocities and τ values were determined from a nonlinear least-squares fit to the nonlinear progress curve for a single-exponential decay at each concentration.

system for the appearance of cooperativity and/or the slow transition. In 30% glycerol, mannose (Table III) behaved similarly to glucose with a slow burst due to a slightly lower $S_{0.5}$ of the initial velocity than the steady state. In both cases, positive cooperativity (n_H = 1.63 and 1.3) was observed only in the steady state and not in the initial velocity. The half-time for the transition appeared to be a slightly faster than with glucose. In the absence of glycerol, mannose was linear and cooperative (Cardenas et al., 1984a; Table II).

The substrate 2-deoxyglucose was similar to glucose in 30% glycerol with, perhaps, a lesser degree of steady-state cooperativity (n_H = 1.2). In the absence of glycerol, however, no cooperativity was observed with 2-deoxyglucose (Cardenas et al., 1979, 1984a; Table III, top), and there was just a suggestion of a transition that was too fast to allow facile measurement of the initial velocity.

With fructose in glycerol, glucokinase displayed a burst that was in the same time range as that with glucose and that also had cooperativity in the steady state. The cooperativity observed in the initial velocity (Table III) may be due to inadequate extrapolation back to zero time or to some as yet unexplained phenomenon. Interestingly, the V_{max} of fructose in glycerol is nearly the same as glucose without the additive. Fructose was surprising in that with care we were also able to observe a slow transition in the dilute buffer (Table III, top) with similar properties. In either solution, the burst at low fructose concentration was primarily due to the change from no cooperativity to positive cooperativity in going from the initial to the steady-state velocity.

Discussion

Glucokinase progress curves were initially observed to undergo a "burst" type process under assay conditions containing 30% glycerol (Figure 1, top curve). If this burst were due to a glucose-induced transition, removal of glucose from the storage buffer should produce a "lag" type transition to the same steady state. The prediction was verified (Figure 1, lower curve) and also demonstrated that the process is reversible with glucose concentration. This hysteresis has now been shown not to be due to an artifactual process such as the effects of changing concentrations of substrates or products, to protein association, decay, or contamination. Instead, the transition is most easily interpreted as being related to a glucose-mediated conformational change in glucokinase. Such a phenomenon can result in kinetic cooperativity of the steady state (Neet & Ainslie, 1980; Cornish-Bowden & Cardenas, 1987).

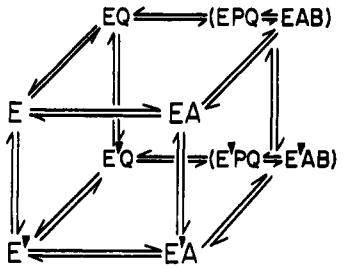

FIGURE 5: Generalized slow transition mechanism for glucokinase. In this model, A = glucose, B = MgATP, P = MgADP, and Q = glucose 6-phosphate. Addition of substrate and release of product are not explicitly shown. The pathways connecting the upper (E) forms to the lower (E′) forms are slow steps compared with the rate of the catalytic cycles. The diagram is drawn as an ordered mechanism to be consistent with other data (see Discussion) and for clarity.

Glycerol has a generalized protective effect on proteins, because of its effect on solvent structure (Tanford, 1965; Shifrin & Parrott, 1975; Gekko & Timasheff, 1981a,b). The polyhydric alcohol may thus preferentially stabilize one enzyme conformation relative to another. Glycerol may also retard the rate of conformational changes in proteins by increasing the microviscosity of the solution (Hardy & Kirsch, 1984). Finally, glycerol may also act to alter the kinetic constants of one enzyme cycle and make the transition more easily visible because of the difference in initial and steady-state velocities. Although glycerol has quantitative effects on the kinetic parameters, i.e., V_{max} and $S_{0.5}$ are decreased 2–4-fold (Table I; Pollard-Knight et al., 1985), it has been shown (Gregoriou et al., 1981) that the kinetic mechanism, determined by isotope exchange patterns, is not affected by glycerol at 30%.

The slow transition mechanism for an ordered reaction mechanism (Figure 5) can account for the kinetic behavior of glucokinase in 30% glycerol and, by inference, in normal buffer. When glucokinase is stored in a buffer containing 50 mM glucose, the glucose in the preincubation would bind the form of glucokinase, e.g., E, with the higher glucose affinity and shift the glucokinase toward that form. At the start of the reaction, essentially all of the glucokinase is saturated with glucose and is in the "high glucose affinity form" (the EA form here). In the initial velocity of the assay, extrapolated to zero time by the computer fit, the upper (E) pathway is predominantly utilized, since the time of the transition from the upper to the lower cycle is longer than the catalytic cycle. The distribution between the pathways will then shift over time, creating the observed nonlinear, hysteretic progress curve. In this case, a kinetic burst occurs as glucokinase goes from a predominantly high-glucose affinity form to a low-affinity form driven by the lower glucose concentration. At higher glucose concentrations in the assay, glucokinase is maintained in the initial form, and the burst is no longer seen (Figure 3). When the reaction is started with glucokinase preincubated in the absence of glucose; the initial distribution of the enzyme depends solely on the E ⇌ E′ equilibrium, and a lag occurs as

the early turnovers are mainly through the lower, E′, lower affinity cycle. Note that the maximal velocity in both v_i and v_{ss} is the same (Figure 3 and Table I), and the progress curve becomes linear at high glucose concentrations. A similar transient may occur without added glycerol, but at too fast a rate to be seen in the normal assay.

Since the ligand (glucose) controls the steady-state distribution between enzyme forms and since the distribution can shift in a nonhyperbolic manner, kinetic cooperativity with respect to either glucose or MgATP occurs in the steady-state velocities (e.g., Table I, row 4). The lowering of the apparent $S_{0.5}$ for glucose as the MgATP concentration decreases is a result of the shift from the sigmoidal to a hyperbolic curve, i.e., the dominance of the low K_m cycle, and has been previously reported in dilute buffer (Storer & Cornish-Bowden, 1976; Cardenas et al., 1979). The positive cooperativity with glucose seen in the steady-state velocities was modified by the MgATP concentration (Table I), and the negative cooperativity with MgATP in the steady states was only seen at low glucose concentrations (Figure 4). It should be emphasized that cooperativity in the steady state does not depend on the initial form of the enzyme but only on the experimental conditions and the cyclical interconversion that continuously occur in the steady state. The increase in glucose cooperativity with increasing concentrations of MgATP can be explained with the slow transition mechanism as with the mnemonic mechanism (Storer & Cornish-Bowden, 1977; Cornish-Bowden & Cardenas, 1987). The slow step of the mechanism (Figure 5) is postulated to be faster than the rate of MgATP binding at low concentrations of MgATP such that at low MgATP concentrations the two forms of glucose–enzyme are near equilibrium, the steady-state shift is abolished, and cooperativity by this mechanism ceases (Storer & Cornish-Bowden, 1977). At high MgATP concentrations, MgATP is more rapidly added to the glucose–enzyme complex, the steady-state distribution is affected, and cooperativity exists. The reciprocal relationship with binding of low concentrations of glucose to free E or E′ as MgATP concentration is varied accounts for the observed effect of glucose on MgATP negative cooperativity in 30% glycerol.

For each of the alternate sugar substrates (Table III), different kinetic parameters appear to be affected; i.e., a different conformational change may be induced with each substrate. With the exception of the fructose burst, the properties in normal buffer solutions, reported here, are similar to those previously reported by Cardenas et al. (1979, 1984a,b). The steady state in glycerol with the alternate substrates resembled the normal assays without glycerol. A primary effect of the added glycerol in most instances was to slow the observed transition. The ability of glycerol to elicit the slow transition shows that individual monosaccharides have different intrinsic transition rates; i.e., different propensities to induce a slow isomerization. These differences, then, determine whether kinetic cooperativity will occur with each hexose.

The mechanism depicted in Figure 5 also accounts for the observation that the competitive inhibitor *N*-acetylglucosamine reduces the glucose cooperativity in glucokinase to a Hill coefficient near unity in addition to its inhibitory effect (Cardenas et al., 1978, 1979). *N*-Acetylglucosamine could bind one enzymatic form preferentially and thus pull the distribution toward that pathway. This behavior is mirrored in the effects in 30% glycerol where the reduction in cooperativity (Table II) occurs in parallel with elimination of the burst (see Results). The effects of *N*-acetylglucosamine are in contrast to those of another competitive inhibitor, palmitoyl-CoA, which binds both forms of the enzyme equivalently, does not change the Hill coefficient for glucose, and has a hyperbolic (rather than an exponential effect) on the $S_{0.5}$ for glucose (Tippett & Neet, 1982a,b). Preferential binding to an enzyme form can thus have a marked effect on the resultant kinetics.

For the initial velocities, both linear and nonlinear types of behavior have been observed with glucokinase in glycerol. The v_i is Michaelis–Menten (e.g., Table I, row 3) if only one kinetic form of the enzyme exists initially. On the other hand, the initial velocity is heterogeneous if more than one kinetic form of the enzyme is present initially (e.g., low [MgATP], Table I, row 5). The existence of two forms of glucokinase with different glucose affinities is supported by the low $S_{0.5}$ for glucose (1.5 mM) seen in the initial velocities, which corresponds to the high glucose affinity form, E.

The reasons for favoring the slow transition mechanism over the mnemonic mechanism (Ricard et al., 1974) or the steady-state random mechanism (Pettersson, 1986) are based on the time-dependent conversion between these two kinetic states and have been discussed (Cardenas et al., 1984a; Cornish-Bowden & Cardenas, 1987; Neet et al., 1988). Briefly, because the mnemonic mechanism has only a single catalytic cycle, it cannot explain the n_H(glucose) less than 1 for the v_i at low MgATP concentration, i.e., a "heterogeneity" of active conformers before any interconversion has occurred. Also, the simple mnemonic mechanism has no second-order term in the second substrate and therefore is not consistent with the n_H(MgATP) less than 1 in the v_{ss} (Figure 4.) Thus, the ligand-induced slow transition model explains the observed v_i and v_{ss} kinetics and the hysteresis reported here in glycerol more completely than do other mechanisms. This conclusion rigorously applies only to the mechanism in glycerol. Extrapolation to dilute buffers is difficult; however, these studies, along with those of Cardenas et al. (1984a) without glycerol, allow us to hypothesize that the slow transition mechanism may also apply under normal assay conditions.

The slow transition mechanism can adequately explain the dependence of the cooperativity of one substrate or product on the concentration of another or on an inhibitor over a range of rates of the slow isomerization. The main weakness in the slow transition model, in fact, is its generality. With numerous variable parameters, specific predictions are difficult to test, and meaningful fits cannot be easily made to obtain kinetic constants. Our recent observations (Lin and Neet, unpublished results) of a slow, glucose-induced conformational change by fluorescent methods may provide a means of independently determining some of the individual rate constants.

Acknowledgments

We thank Martin C. Meyer for assistance in purification of glucokinase. We also thank Walter K. Vogel, Dr. Choong Kim, and Dr. Sheng-Xeng Lin for helpful discussions and Drs. Nelson Phillips and Joyce Jentoft for reading the manuscript.

Registry No. GlcNAc, 7512-17-6; MgATP, 1476-84-2; glucose, 50-99-7; glycerol, 56-81-5; mannose, 3458-28-4; 2-deoxyglucose, 154-17-6; fructose, 57-48-7; glucokinase, 9001-36-9.

References

Ainslie, G. R., Jr., Shill, J. P., & Neet, K. E. (1972) *J. Biol. Chem. 247*, 7088–7096.

Beck, W. S. (1967) *J. Biol. Chem. 242*, 3148–3158.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Bontemps, F., Hue, L., & Hers, H.-G. (1978) *Biochem. J. 174*, 603–611.

Buc, J., Ricard, J., & Meunier, J. (1977) *Eur. J. Biochem. 80*, 593-601.
Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1978) *Arch. Biochem. Biophys. 190*, 142-148.
Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1979) *Arch. Biol. Med. Exp. 12*, 571-580.
Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1984a) *Eur. J. Biochem. 145*, 163-171.
Cardenas, M. L., Rabajille, E., & Niemeyer, H. (1984b) *Biochem. J. 222*, 363-370.
Cornish-Bowden, A., & Cardenas, M. L. (1987) *J. Theor. Biol. 124*, 1-23.
Frieden, C. (1970) *J. Biol. Chem. 245*, 5788-5799.
Furman, T. C., & Neet, K. E. (1983) *J. Biol. Chem. 258*, 4930-4936.
Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667-4676.
Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677-4684.
Gregoriou, M., Trayer, I. P., & Cornish-Bowden, A. (1981) *Biochemistry 20*, 499-506.
Hardy, L. E., & Kirsch, J. F. (1984) *Biochemistry 23*, 1275-1282.
Holroyde, M. J., Allen, M. B., Storer, A. C., Warsy, A. S., Chesher, J. M. E., Trayer, I. P., Cornish-Bowden, A., & Walker, D. G. (1976) *Biochem. J. 153*, 363-373.
Meglasson, M. D., & Matschinsky, F. M. (1983) *J. Biol. Chem. 258*, 6705-6708.
Neet, K. E., & Ainslie, G. R., Jr. (1980) *Methods Enzymol. 64*, 192-226.
Neet, K. E., Furman, T. C., & Hueston, W. J. (1982) *Arch. Biochem. Biophys. 213*, 14-25.
Neet, K. E., Tippett, P. S., & Keenan, R. P. (1988) in *Dynamics of Solubilized and Immobilized Enzyme Systems* (Chock, B., & Huang, C., Eds.) pp 28-39, Springer-Verlag, Berlin.
Olavarria, J. M., Cardenas, M. L., & Niemeyer, H. (1982) *Arch.Biol. Med. Exp. 15*, 365-369.
Pettersson, G. (1986) *Biochem. J. 233*, 347-350.
Pollard-Knight, D., & Cornish-Bowden, A. (1982) *Mol. Cell. Biochem. 44*, 71-80.
Pollard-Knight, D., Connolly, B. A., Cornish-Bowden, A., & Trayer, I. P. (1985) *Arch. Biochem. Biophys. 237*, 328-334.
Ricard, J., Meunier, J., & Buc, J. (1974) *Eur. J. Biochem. 49*, 195-208.
Shifrin, S., & Parrott, C. L. (1975) *Arch. Biochem. Biophys. 166*, 426-432.
Storer, A. C., & Cornish-Bowden, A. (1976) *Biochem. J. 159*, 7-14.
Storer, A. C., & Cornish-Bowden, A. (1977) *Biochem. J. 165*, 61-69.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122-282.
Tippett, P. S. (1981) Ph.D. Dissertation, Case Western Reserve University.
Tippett, P. S., & Neet, K. E. (1982a) *J. Biol. Chem. 257*, 12839-12845.
Tippett, P. S., & Neet, K. E. (1982b) *J. Biol. Chem. 257*, 12846-12852.
Tippett, P. S., & Neet, K. E. (1983) *Arch. Biochem. Biophys. 222*, 285-298.
Viola, R. E., Morrison, J. F., & Cleland, W. W. (1980) *Biochemistry 19*, 3131-3137.
Womack, F. C., & Colowick, S. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5080-5084.

Quantitative Footprinting Analysis. Binding to a Single Site†

Robert Rehfuss, Jerry Goodisman,* and James C. Dabrowiak*
Department of Chemistry, Syracuse University, Syracuse, New York 13244-1200
Received June 19, 1989; Revised Manuscript Received August 24, 1989

ABSTRACT: The theory for measuring ligand binding constants from footprinting autoradiographic data associated with a single binding site is derived. If the ligand and DNA cleavage agent compete for a common site, the spot intensities are not proportional to the amount of DNA not blocked by ligand. The analysis of a single site is experimentally illustrated by using results for the anticancer drug actinomycin D interacting with the duplex d(TAGCGCTA)$_2$ as probed with the hydrolytic enzyme DNase I.

Footprinting analysis utilizes DNA sequencing methodology to locate the binding sites of drugs and proteins on DNA molecules of heterogeneous sequence (Galas & Schmitz, 1978; Lane et al., 1983; Scamrov & Beabealashvili, 1983; Van Dyke et al., 1982). In the footprinting experiment, DNA which has come to equilibrium with a ligand is allowed to interact with a probe capable of cutting the DNA at various sites. From the amounts of the different oligonucleotide fragments produced, one can infer where the ligand binds on the DNA, assuming that ligand bound at a DNA site prevents the probe from cleaving at that site. In quantitative footprinting studies, the amounts of fragments produced in the digest are analyzed to extract individual site binding isotherms and determine ligand binding constants as a function of sequence. It has now been demonstrated that one can obtain binding constants for ligands on large DNA polymers as well as small oligomers (Brenowitz et al., 1986; Carey, 1988; Dabrowiak & Goodisman, 1989; Goodisman & Dabrowiak, 1990; Ikeda & Richardson, 1986; Gunderson et al., 1987; Letovsky & Dynan, 1989; Fish et al., 1988; Brenowitz & Senear, 1989).

For an oligomer with only one binding site, the quantitative analysis of footprinting data is quite simple, but not without pitfalls. In the present paper, we show the correct method for extracting the ligand–DNA binding constant from footprinting

† Supported by American Cancer Society Grant NP-681.

0006-2960/90/0429-777$02.50/0