An Allosteric Model for the Inhibition of Glucokinase by Long Chain Acyl Coenzyme A

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Rat liver glucokinase is specifically inhibited by palmitoyl-CoA and other long chain acyl-CoAs at concentrations well below their critical micelle concentrations (Tippett, P. S., and Neet, K. E. (1982) J. Biol. Chem. 257, 12839–12848). Kinetic studies of this inhibition indicate that the long chain acyl-CoAs act on glucokinase through an allosteric site. The high Mg" concentration is competitive with both glucose and MgATP at either saturating or half-maximal fixed substrate concentrations and the apparent K, is the same (1.8 μM for palmitoyl-CoA) with respect to either substrate. The positive cooperativity which glucokinase normally displays with glucose is unaffected by the inhibition. Palmitoyl-CoA (as well as the other long chain acyl-CoAs) inhibition is itself positively cooperative, yet this cooperativity is unchanged (Hill coefficient = 1.78 ± 0.3 (n = 32) over a full range of concentrations of either substrate.

Inhibition by arachidoyl-CoA in an assay mix containing low concentrations of free magnesium did not continue toward zero activity with increasing concentrations of inhibitor but instead reached a limiting value. This leveling off was not due to the formation of arachidonate but instead reached a limiting concentration of inhibitor or a high MgCl₂ concentration or a high M, concentration by the addition of 30% glycerol. Data for this limited inhibition of glucokinase was limited and did not approach zero activity at infinite inhibitor concentrations. Thus, classical competitive inhibition did not occur.

The inhibition of glucokinase by all long chain acyl-CoAs was limited and did not approach zero activity at infinite inhibitor concentrations. Thus, classical competitive inhibition did not occur.

Careful examination of the kinetics of the inhibition, especially of arachidonyl- and palmitoyl-CoA under several conditions, suggests that a long chain acyl-CoA may bind at an allosteric site on the glucokinase monomer and that the extent of the inhibition is determined by both its binding constant (K,) and the extent of the conformational change induced on binding (α).

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2 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 a degree of Doctor of Philosophy.

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1 The abbreviations used are: S, substrate concentration for half-maximal activity; n, Hill coefficient; PEP, phosphoenolpyruvate; cme, critical micelle concentration; S, substrate concentration; I, inhibitor concentration; X, concentration of second ligand (palmitoyl-CoA here); α, a factor which is equal to the maximal fold increase in the S, or K, when the inhibitor or substrate sites, respectively, are saturated.


**Materials and Methods**

**Results and Discussion**

**Kinetics of Inhibition by Palmitoyl-CoA in Low Free MgCl₂**—We have shown previously (12) that both the normal positive cooperativity which glucokinase displays toward glucose (the upward curvature in Fig. 1A) at high concentrations of ATP and the $V_{\text{max}}$ (with saturating ATP) are unaffected at all levels of inhibition by palmitoyl-CoA. Since the $K_{o}$ for glucose is the only parameter affected by palmitoyl-CoA, the inhibition is competitive with respect to glucose (Fig. 1A).

Fig. 1B shows double reciprocal plots at saturating fixed glucose with MgATP as the varied substrate at several inhibitory concentrations of palmitoyl-CoA. At all levels of inhibition, glucokinase retains its linear (Michaelis-Menten) kinetics with MgATP (16). Furthermore, the inhibition by palmitoyl-CoA is competitive with MgATP, which is surprising since palmitoyl-CoA is also competitive with glucose, the other substrate (Fig. 1A). The competitive nature of this inhibition was confirmed essentially with any other substrate at saturating levels as shown in Fig. 1, (100 mM for glucose or 5 mM for MgATP) or at half-maximal levels (5 mM for glucose or 0.5 mM for MgATP) (Fig. 2). The nearly linear kinetics with glucose at half-saturating ATP (Fig. 2A; compare with Fig. 1A) is normal (the Hill coefficient for glucose is dependent on ATP concentrations) (16) and the linearity is unaffected by the palmitoyl-CoA inhibition. Dixon plots for these data (Figs. 1C, 1D, 2C, and 2D) reveal that the inhibition by palmitoyl-CoA itself shows positive cooperativity, whether the varied substrate is glucose (Fig. 1C) or ATP (Fig. 1D).

Furthermore, Hill plots (not shown) of these data indicate that the apparent cooperativity in the inhibition does not change with different constant substrate concentrations. With ATP as the changing fixed substrate (Fig. 1D), the plots gave a median Hill coefficient for palmitoyl-CoA of 1.77 ± 0.25, while with various fixed glucose concentrations (Fig. 1C), the Hill coefficient for the inhibition by palmitoyl-CoA was 1.67 ± 0.16. The Hill coefficients were not different if the concentration of the constant substrate was reduced to the $S_{0.5}$ value (Fig. 2, C and D). With 5 mM glucose as the constant substrate, the median Hill coefficient was 1.86 ± 0.51, while with 0.5 mM MgATP constant and glucose and palmitoyl-CoA varied, the Hill coefficient was 1.71 ± 0.13. Thus, within experimental error, the extent of cooperativity in the inhibition by palmitoyl-CoA was invariant over a wide range of concentrations of both substrates and gave an overall median value of 1.75 ± 0.26 ($n$ = 32).

No attempt was made to define all of the factors which affected the extent of this cooperativity with palmitoyl-CoA, but the pH of the assay did have a pronounced effect. The Hill coefficient calculated for inhibition at pH 6.5 was 1.73 ($n$ = 2) and at pH 7.5 was 1.75 ($n$ = 32), but at pH 8.5, the Dixon plots gave a median Hill coefficient of only 1.17 ($n$ = 2) (data not shown) with little change in $K_{o}$.

The apparent $K_{o}$ (from Figs. 1 and 2) had essentially equivalent values either estimated by determining the palmitoyl-CoA concentration required to double the $S_{0.5}$ for MgATP (or double the $S_{0.5}$ for glucose) or estimated by plotting apparent $K_{o}$ versus MgATP or glucose and extrapolating to zero concentration of the variable substrate (at a fixed concentration of the substrate). The apparent $K_{o}$ for palmitoyl-CoA: (a) determined versus glucose at saturating MgATP was 1.67 ± 0.18 mM; (b) determined versus MgATP at saturating glucose was 1.95 ± 0.35 mM; (c) determined versus glucose at half-maximal MgATP was 0.71 ± 0.44 mM; and (d) determined versus MgATP at half-maximal glucose was 0.68 ± 0.22 mM. In other words, the apparent $K_{o}$ (competitive) for palmitoyl-CoA was not dependent on which substrate was the variable one (compare values $a$ and $b$ since values $c$ and $d$ above) at either saturating or half-maximal concentrations of the fixed substrate.

Thus, in the low magnesium system where the inhibition by palmitoyl-CoA was complete (12), the addition of this inhibitor served only to increase the $K_{o}$ or $S_{0.5}$ of the substrates; the increase in the $K_{o}$ for ATP was proportional to the increase in the $S_{0.5}$ for glucose for a given concentration of inhibitor. There was no effect of the inhibitor on either the $V_{\text{max}}$ or substrate cooperativity and no effect of the substrates on the cooperativity of the inhibition.

**Inhibition by Arachidoyl-CoA**—In contrast to the complete inhibition in the low magnesium system, inhibition of glucokinase by lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, or arachidoyl-CoA was previously shown to reach a limit, dependent on the particular ligand, when the assays were performed in 5 mM or greater free magnesium (excess over ATP) concentrations (12). This partial competitive nature of the inhibition was not apparent in assays with equimolar MgCl₂ and ATP where complete inhibition could be approached at high inhibitor concentration (Figs. 1 and 2). Because the critical micelle concentration was close to the point where the inhibition reached its limit in the high magnesium system, and because the cmc could not be significantly altered by other means in the presence of excess Mg²⁺, it was not possible to determine with certainty whether this "levelling off" was due to the formation of noninhibitory micelles or to some other property of the inhibition by these acyl-CoAs inherent with glucokinase.

In the high magnesium system assays, where the inhibition by all acyl-CoAs was limited (12), arachidonyl-CoA had no significant effect on glucokinase activity (Fig. 3). In the low magnesium system where the other acyl-CoAs were fully inhibitory, however, inhibition by arachidonyl-CoA was limited (Fig. 4, solid lines) in a manner similar to the behavior of the other acyl-CoAs in the high Mg²⁺ assays. The cmc for arachidonyl-CoA in the low Mg²⁺ assay mixture (0.3 mM) is indicated by an arrow (Fig. 4). In this low Mg²⁺ system, it was possible to increase the cmc of palmitoyl-CoA over 4-fold by the inclusion of 30% glycerol in the assay mixture (12). Similarly, the cmc of arachidonyl-CoA measured by pinacyanol chloride dye incorporation in a low Mg²⁺ assay mix containing 30% glycerol was increased from 0.3 to 1.8 μM (data not shown). If the leveling off of this inhibition by arachidonyl-CoA in the low Mg²⁺ mix (Fig. 4) was due to the formation of arachidonyl-CoA micelles and the resultant limit on the attainable concentration of the free (monomeric) form of arachidonyl-CoA, then the extent of this inhibition should be greater and the inhibition limit increased or eliminated if the cmc for arachidonyl-CoA were increased. In fact, inhibition of glucokinase by arachidonyl-CoA in the presence of 30% glycerol was nearly identical with the inhibition shown in Fig. 4. In a 2 mM glucose, 5 mM MgCl₂, 5 mM ATP, 30% glycerol series, the maximum extent of inhibition was only 53% (compared with 48% in the absence of glycerol (Fig. 4)), even though the cmc for arachidonyl-CoA had been increased approximately 6-fold. The formation of micelles therefore is not the cause of the inhibition limit, at least for arachidonyl-CoA.
Allosteric Inhibition of Glucokinase

Proposed Allosteric Acyl-CoA Site—Much of the data accumulated for the inhibition of glucokinase by the long chain acyl-CoAs do not support the notion of the inhibitor binding to the substrate site or sites, competing directly with the substrate or substrates, and therefore giving rise to kinetic competitive inhibition patterns. The postulate of a separate, allosteric site for the acyl-CoAs on glucokinase is more tenable. The proof of the existence of an allosteric site is difficult without physical separation or functional isolation of it from the active site, but there are several lines of evidence which support the allosteric proposal.

First, competitive inhibition patterns with respect to both substrates (Figs. 1 and 2) are rare and are difficult to explain mechanistically in terms of classical competitive binding at the substrate site(s). Glucokinase probably follows a compulsory ordered mechanism (17), like several other hexokinases (18), with glucose the first substrate to bind and glucose 6-phosphate the last product to be released. In this case, if palmitoyl-CoA bound at the glucose site, inhibition would be competitive with glucose and mixed with ATP. Conversely, if it bound at the ATP site, inhibition would be competitive with ATP and mixed with glucose. If it acted at both sites, there would be a component of mixed inhibition with respect to each substrate. Recourse to a random mechanism, to the proper conformation (4, 19) or slow transition (20, 21) mechanism for glucokinase with four different enzyme forms (13), or to any mechanism in which palmitoyl-CoA binds to either the glucose site or the ATP site would also give some component of mixed inhibition with respect to at least one of the substrates.

The inhibition patterns could be competitive with respect to both substrates if palmitoyl-CoA bound at an allosteric site, induced a conformational change in the enzyme causing a decreased binding affinity for both substrates, and had no effect on the catalytic capacity (V_{max}) of glucokinase. A rapid equilibrium between the free enzyme (E) and the enzyme-inhibitor complex (EI) could both eliminate any mixed component from the inhibition and allow the inhibition to span a full range of apparent substrate S_{0.5} values, because the concentration of E determines the equilibrium between the E and EI forms.

Second, the apparent K, for the inhibition by palmitoyl-CoA is virtually the same (1.67 versus 1.96 μM) whether glucose or ATP is saturating and is also the same (0.69 versus 0.71 μM) whether glucose or ATP is fixed at its half-maximal level. Although it would be possible for the inhibitor to bind to the ATP site at saturating or half-maximal glucose with the same affinity as it binds to the glucose site at saturating or half-maximal ATP concentrations, respectively, a more likely situation would be one in which the inhibitor binds the same allosteric site in each case, especially since the inhibitor, long chain acyl-CoA, bears virtually no structural resemblance to glucose.

Third, if the inhibitor actually binds at the glucose site (causing the competitive inhibition with glucose), then the extent of cooperativity that glucokinase normally displays toward glucose would necessarily be altered at various levels of inhibition. Monomeric glucokinase very likely derives its glucose cooperativity through a mnemonic (4, 19) or slow transition (13, 20, 21) mechanism. The cooperativity is dependent on two interchangeable forms of the enzyme monomer which bind glucose with different affinities; the cooperativity is then generated because changing the glucose concentrations alters the steady state distribution between forms. The binding of an inhibitor at the glucose site would necessarily affect the cooperativity by binding differentially and pulling the distribution toward one form or another. A true competitive inhibitor, N-acetylglucosamine, does eliminate the glucose cooperativity in glucokinase (19, 22). Only if the inhibitor bound equally to the different forms (which have different glucose affinities) could the cooperativity possibly remain unchanged.

Fourth, it can be seen that the inhibition of glucokinase by palmitoyl-CoA is itself positively cooperative (Figs. 1 and 2). If this cooperativity were due to binding of palmitoyl-CoA to the two substrate sites, the degree of cooperativity would necessarily be dependent on the substrate concentration. Yet the extent of the cooperativity was invariant whether the second substrate was held at saturating or half-maximal levels or varied over a full range of concentrations (ie, at 32 different combinations of glucose and ATP concentrations).

Finally, although the limited inhibition seen with the C-12 through C-18 CoAs (12) in the high magnesium assay system could perhaps have been rationalized by the formation of micelles at a critical concentration which stopped any further increase in the free concentration of amphiphile, this is clearly not the case for the limited inhibition seen with arachidoyl-CoA, since the cmc could be varied over a 6-fold range without changing the limit of inhibition. Such partial competition is more consistent with allosteric inhibition than with isosteric inhibition.

Taken together, these points strongly suggest that the binding sites or sites for palmitoyl-CoA on glucokinase are allosteric, physically separate from the catalytic glucose and MgATP sites.

The Miniprint contains a simple mathematical model for an allosteric acyl-CoA site in glucokinase, some proposed theoretical consequences of this model, several experimental results in accord with predicted behavior, and model-derived kinetic parameters for all the long chain acyl-CoAs studied in both high and low magnesium conditions. The allosteric model easily explains all of the observed behavior between fatty acyl-CoAs and glucokinase under all conditions thus far examined.

Physiological Significance—The full physiological implications of this allosteric model for glucokinase remain to be determined. It is clear, however, that in the case of the long chain acyl-CoAs, an effective inhibitor must have both a K appropriate to its concentration in vivo and a high enough α to translate that binding into meaningful inhibition. For glucokinase and the long chain acyl-CoAs, the two most abundant forms in vivo, palmitoyl- and oleoyl-CoA, have the most appropriate inhibition parameters. The K values are probably within the physiological range of free acyl-CoA (as discussed in Ref. 13) and the α terms are high. For the longer saturated acyl-CoAs, stearoyl- and arachidoyl-CoA, the decreased α begins to limit the extent of inhibition and for the shorter analogues, myristoyl- and lauroyl-CoA, the K is probably well above the physiological range.

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REFERENCES
Allosteric Inhibition of Glucokinase

Allosteric Inhibition of Glucokinase

**Materials and Methods**

- **Materials**
  - ATP, NAD, PEP, and glucose-6-phosphate dehydrogenase (G6PDH) were from Sigma. All other compounds were the best grade available and were obtained from either Sigma or Fisher.

- **Methods**
  - Glucokinase was prepared by modification (12) of the method of Hildreth et al. (13). Glucokinase was normally assayed at 25°C with a glucose-6-phosphate dehydrogenase coupled system (12, 14). The standard assay mixture was, in ml: 100 mM Tris-acetate, pH 7.5, 5 mM NAD, 1 mM dithiothreitol, 1 mM ATP, 3 units of G6PDH, and glucose as indicated.

  - The concentrations of all the CoA esters were determined by their absorbance at 237 nm with an extinction coefficient of 13,600 M⁻¹ cm⁻¹. The rate of formation of CoA was measured by the phoshoenolpyruvate method (15) as described previously (12). The experiments were done using 10 mM MgCl₂ concentrations. All other concentrations were identical. All the other parameters, e.g., substrate and inhibitor concentrations, and methods for glucose and CoA, were performed independently or were defined by the experiment.

**Computer Simulations**

- Model fitting was performed on a Hewlett-Packard model 9815A desktop calculator and plotted on a Hewlett-Packard model 1225A plotter. All other parameters were assigned. All the other parameters, e.g., substrate and inhibitor concentrations, and methods for glucose and CoA, were performed independently or were defined by the experiment.
Allosteric Inhibition of Glucokinase

Glucokinase is a key enzyme in the regulation of blood glucose levels. It catalyzes the phosphorylation of glucose, and its activity is modulated by allosteric inhibitors. The inhibition of glucokinase by allosteric inhibitors can be described by the following equation:

\[ \text{Glucokinase} + \text{Inhibitor} \rightarrow \text{Inhibited Glucokinase} \]

The inhibition constant, \( K_i \), can be determined using the Lineweaver-Burk plot. The plots for different allosteric inhibitors are shown below. The data points are plotted on a double reciprocal plot, and the inhibition constant, \( K_i \), is determined from the slope of the line.

**Table 1:**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( K_i ) (mM)</th>
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<tr>
<td>Acetate</td>
<td>0.05</td>
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<tr>
<td>Malonate</td>
<td>0.01</td>
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<td>PMS</td>
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**Figure 1:**

A double reciprocal plot showing the inhibition of glucokinase by different allosteric inhibitors. The plots are linear, indicating competitive inhibition. The inhibition constants \( K_i \) are determined from the slopes of the lines.

Although the possibility of a co-reaction between the inhibitor and the enzyme (I), and the extent of the conformational change induced in the enzyme (II) should be possible to take into account, the difference between a co-reaction and a partial competitive inhibition was less clear for palmitoyl CoA in the high magnesium system (II). However, the other arguments for an allosteric site hold for all in the long chain acyl CoA. Theoretical curves for palmitoyl CoA in high Mg\(^{2+}\) conditions at several glucose concentrations using previously determined values of \( K_i \) (5) and \( m_i \) coefficient correlate well with the experimental points at an assigned of 0.5 for palmitoyl CoA (Fig. 5). Curves fitting was performed on all the long chain acyl CoA (and here). The best set of theoretical curves are given in Table 1 for these ligands in both the high and low magnesium systems.

The molecular basis presented here for the phenomenological observation of allosteric CoA's effect on glucokinase (Eq. 2 in the Introduction) is based upon a model for kinetic cooperativity in which the enymes exist as two catalytic states, slowly interconvertible through a rate-determining conformational change (22, 23). This model has been extended to include rate-dependent cooperativity (24) and to account for partial cooperative activation of glucokinase (10, 24). We have demonstrated that the pharmacological properties of palmitoyl CoA (25, 26) among the enzymes are due to the following factors: for an enzyme bound to a substrate and for an enzyme bound to a substrate of a given enzyme. Theoretical curves fit the data well and show the expected sigmoidal shape. Kinetic positive cooperativity of palmitoyl CoA (27) as a substrate is observed. The rate constants for the co-reactions are enzymatic rate equation, or to two or more acyl CoA binding sites per monomer.

With the values for \( k, M_i \), for palmitoyl CoA in the low magnesium system (Table 1) (palmitoyl CoA was adjusted to a of 1800), theoretical curves for co-reaction inhibition were generated for a wide range of conditions. Two of the curves, for \( k \) and \( M_i \), are representative (Fig. 6). At very low \( M_i \) and \( k \) values, the inhibition is competitive, while at high \( M_i \) and low \( k \) values, the inhibition becomes non-competitive and the rate constant drops rapidly with increasing substrate concentration. The inhibition constants for palmitoyl CoA (25, 26) are also listed in Table 1, and the theoretical curves are shown in Figure 6. At very low \( M_i \) and high \( k \) values, the inhibition is still competitive, while at high \( M_i \) and low \( k \) values, the inhibition becomes non-competitive and the rate constant drops rapidly with increasing substrate concentration. The inhibition constants for palmitoyl CoA (25, 26) are also listed in Table 1, and the theoretical curves are shown in Figure 6.

Activation with a lowering of the \( k_i \) of the inhibitor is more likely to be observed in the presence of a high Mg\(^{2+}\) system, where palmitoyl CoA activation is activated by the addition of Mg\(^{2+}\) ions. The apparent activation of palmitoyl CoA from its free form, which is an inhibitor, is a greater reduced ability to induce the inhibitor conformational change. A closer to unity.

In conclusion, allosteric inhibitors of glucokinase can be classified as competitive or non-competitive, depending on the nature of the inhibitor and the experimental conditions. The inhibitory effects of palmitoyl CoA on glucokinase activity can be explained by a mechanism involving co-reaction. Theoretical curves for co-reaction inhibition were generated for a wide range of conditions. The results show that at low Mg\(^{2+}\) concentrations, the inhibition becomes non-competitive, while at high Mg\(^{2+}\) concentrations, the inhibition remains competitive. The inhibition constants for palmitoyl CoA are also listed in Table 1, and the theoretical curves are shown in Figure 6.
Allosteric Inhibition of Glucokinase

Allosteric inhibition of glucokinase activity in the high magnesium system (Fig. 3). The technique is illustrated by measuring the effect of 25 mM glucose on the activity of the enzyme in the presence of various concentrations of acetyl CoA. The results show that the inhibition is concentration-dependent and that the inhibition constant (K_i) is approximately 0.6 mM for glucose. The inhibition is reversible, with the enzyme activity returning to control levels when the glucose is removed from the reaction mixture. The inhibition constant for acetyl CoA is approximately 0.3 mM, indicating that the inhibition is specific for this substrate.

The inhibition constant (K_i) for glucose is used to calculate the affinity of the enzyme for the substrate. The lower the K_i, the higher the affinity of the enzyme for the substrate. In this case, the K_i for glucose is 0.6 mM, indicating that the enzyme has a relatively high affinity for glucose.

The inhibition of glucokinase by acetyl CoA is not specific for glucose, as the enzyme is inhibited by other acyl-CoA analogues, such as palmitoyl CoA. The inhibition by palmitoyl CoA is concentration-dependent, with an inhibition constant of approximately 1.2 mM. The inhibition by palmitoyl CoA is specific, as the enzyme is not inhibited by other substrates, such as fructose-6-phosphate or glyceraldehyde-3-phosphate.