# Determination of the Screw Sense Specificity of Bovine Liver Fructokinase†

Vincent L. Pecoraro,‡ Alan R. Rendina,§ and W. W. Cleland*
*Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706*
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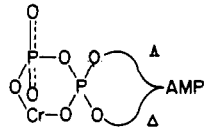
ABSTRACT: Fructokinase from beef liver showed a clear reversal in specificity when the two isomers of ATPβS were used as substrates with $Mg^{2+}$ and $Cd^{2+}$, with the $S_P$ isomer having the higher $V/K$ value with $Mg^{2+}$ and the $R_P$ isomer the higher value with $Cd^{2+}$. The Δ isomer of MgATP is thus the active form of the substrate. The substitution of sulfur for oxygen in the noncoordinated position of the β-phosphate caused a 102-fold decrease in $V/K$ over the value seen with MgATP, while substitution in the coordinated position gave a 21-fold decrease over the $V/K$ value seen with CdATP. The $K_m$ values were little affected by sulfur substitution, showing that the wrong screw sense isomers were nonproductively bound almost as well as the correct ones. When ADPαS was used as a substrate in the reverse reaction, the $S_P$ isomer showed the highest $V/K$ value with both $Mg^{2+}$ and $Cd^{2+}$, suggesting that the metal ion is not coordinated to the α-phosphate during transphosphorylation. The failure of CrATP to act as a substrate for fructokinase suggests that the enzyme inserts one of its side chains into the inner coordination sphere of the metal ion during the reaction.

Fructokinase (ATP:D-fructose 1-phosphotransferase, EC 2.7.1.3) uses MgATP to phosphorylate β-D-fructofuranose and a number of closely related substrates having a furanose ring and the β-D or α-L configuration at C-2 (Raushel & Cleland, 1977):

$$\text{fructose} + \text{MgATP} \rightleftharpoons \text{MgADP} + \text{fructose 1-phosphate} \quad (1)$$

The nucleotide requirement is much stricter, with only ATP, 2′-dATP, and 3′-dATP functioning as phosphate donors (Parks et al., 1957; Adelman et al., 1967). MgATP can exist as a number of coordination isomers, and Cornelius & Cleland (1978) and Dunaway-Mariano & Cleland (1980) developed the use of inert coordination complexes of Co(III) or Cr(III) of known stereochemistry to determine which was the substrate for a given enzyme. They found that a number of enzymes used either the Λ- or Δ-bidentate complex of CrATP as a substrate:[1]




However, this method works only when the enzyme does not have to insert one of its side chains as a ligand in the inner coordination sphere of the nucleotide-bound metal ion during the reaction. While phosphofructokinase was found to show substrate activity with Δ-bidentate CrATP (Dunaway-Mariano & Cleland, 1980), we have failed to find any substrate activity with beef liver fructokinase, although CrATP was bound to the enzyme with the same affinity as MgATP (Raushel & Cleland, 1977).

As a result, we have turned to the use of chiral nucleoside phosphorothioates with $Mg^{2+}$ and $Cd^{2+}$ as the metal ions to determine the screw sense specificity of fructokinase for MgATP isomers. This method, pioneered by Eckstein (1975) and Cohn (1982), depends on the preference of $Mg^{2+}$ for coordination of oxygen rather than sulfur and the reverse preference of $Cd^{2+}$. Our recent determination of exact values for these preferences (Pecoraro et al., 1984) has permitted us to determine not only the screw sense specificity of fructokinase but also the quantitative effects of sulfur substitution in the coordinated and noncoordinated positions of the metal–ATP complex on binding strength and catalytic rate.

## Materials and Methods

Fructokinase (14 units/mg of protein) was isolated from beef liver by the method of Raushel & Cleland (1977). A myokinase impurity was removed by passage of the enzyme solution through a 1 × 10 cm blue Sepharose column which was eluted with 20 mM imidazole, pH 7, containing 1 mM dithiothreitol and 0.1 mM ethylenediaminetetraacetic acid (EDTA)[2] at 4 °C. Fructokinase is not retained, but myokinase is. All other enzymes, phosphoenolpyruvate, ATP, ADP, and NADH were from Boehringer-Mannheim, except sorbitol dehydrogenase which was from Sigma. ATPβS and ADPαS (the latter supplied by Dr. P. A. Frey) were synthesized by the method of Eckstein & Goody (1976). Trace amounts of the $R_P$ isomer of ATPβS were removed from ($S_P$)-ATPβS by incubation with hexokinase and rechromatography on DEAE-Sephadex. Magnesium metal (Fischer) was dissolved in $HNO_3$ to prepare a stock solution of $Mg^{2+}$, while $CdCl_2$ (Baker) was used as the source of $Cd^{2+}$. Both metal ion solutions were standardized by titration with EDTA using Eriochrome Black T as the indicator as described by Welcher (1958). The concentrations of solutions of $Na_2ATP$ and NaADP were determined by enzymatic end point assay, with a hexokinase–glucose-6-P dehydrogenase assay for ATP and a pyruvate kinase–lactate dehydrogenase assay for ADP, assuming that ε for the reduced nucleotides was 6200 at 340 nm. The thionucleotides were converted from the triethylammonium salt to the $K^+$ form by making a slurry with Dowex-50-$H^+$ at 4 °C followed by filtration and readjustment to neutral pH with KOH, and their concentrations were determined spectrally at 259 nm, assuming ε = 15 400. $^{31}P$

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‡Present address: Department of Chemistry, University of Michigan, Ann Arbor, MI 48109.
§Present address: Chevron Chemical Co., Richmond, CA 94804.

[1] The screw sense nomenclature is that of Cornelius & Cleland (1978) in which an axis through the metal ion perpendicular to the chelate ring and the bond from the chelate ring to the rest of the molecule define the helical sense.

[2] Abbreviations: ATPβS and ADPαS, nucleotides substituted with sulfur in the indicated position; EDTA, ethylenediaminetetraacetic acid.

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Table I: Kinetic Constants for Phosphorylation of Fructose[a]

| metal ion | substrate | screw[b] sense | relative $V$ | relative[c] $V/K$ | $K_m$ (mM) |
|---|---|---|---|---|---|
| Mg | ATP | | (100) | 197 ± 14 | 0.51 ± 0.06 |
| Cd | ATP | | 55.6 ± 5.3 | 156 ± 26 | 0.36 ± 0.09 |
| Mg | ($R_P$)-ATPβS | Λ | 0.40 ± 0.10 | 0.23 ± 0.03 | 1.73 ± 0.58 |
| Cd | ($R_P$)-ATPβS | Δ | 9.09 ± 2.0 (9.0) | 7.51 ± 0.87 (7.43) | 1.21 ± 0.33 |
| Mg | ($S_P$)-ATPβS | Δ | 4.42 ± 0.82 (4.14) | 2.06 ± 0.23 (1.93) | 2.14 ± 0.60 |
| Cd | ($S_P$)-ATPβS | Λ | 0.29 ± 0.07 | 0.16 ± 0.03 | 1.81 ± 0.56 |

[a] Data obtained at 25 °C, in 100 mM KCl, 0.1 M Hepes, pH 8, and 15 mM fructose, with the pyruvate kinase–lactate dehydrogenase coupled system. The values of relative $V$ and $V/K$ in parentheses are corrected for contaminating ATP as described in the text. [b] Screw sense of the major coordination isomer present in solution. [c] Relative $V$ divided by $K_m$.

NMR spectra were obtained for both diastereomers of ATPβS and ADPαS to ensure that they were pure.

*Enzymatic Assays.* All assays were run at pH 8, 25 °C, in 0.1 M $K^+$-Hepes. The concentration of free $Mg^{2+}$ was kept at 5 mM, assuming the stability constants reported by Pecoraro et al. (1984). However, the total level of $Cd^{2+}$ added was always 90% of the total nucleotide concentration present. The actual levels of Cd–nucleotide complexes were then calculated from the known stability constants (Pecoraro et al., 1984).

The activity of fructokinase in the forward direction was assayed in two ways. With $Mg^{2+}$ and $Cd^{2+}$ complexes of ATP and ($R_P$)- or ($S_P$)-ATPβS, a coupled enzymatic assay involving pyruvate kinase, lactate dehydrogenase, and 15 mM fructose was used (although this assay regenerates primarily the $S_P$ isomer of ATPβS, it gives valid results as long as initial velocities are used). With MgATPβS (both isomers), a fixed time assay was also used in which a 0.5-mL aliquot was removed at 1, 5, 10, and 20 min from the reaction mixture and acidified with perchloric acid to pH 2. After 3 drops of $CHCl_3$ was added and the solution vortexed for 15 s, the precipitated protein was filtered off with glass wool and the pH readjusted to 7 with KOH. The solution was filtered to remove $KClO_4$, and the end point assay for ADPβS was carried out with the pyruvate kinase–lactate dehydrogenase couple. The initial rate at each substrate concentration was based on the four time points, with four substrate concentrations being used to define $V$ and $V/K$. To correct for decomposition of the thionucleotide, one set of time points was taken in the absence of fructokinase. The kinetics from the coupled assays were in good agreement with those from the fixed time assays. A 5–10-fold drop in the rate with CdATP was observed when 0.1 mM dithiothreitol was present in the assay mixture; for this reason the above assays did not contain dithiothreitol.

The activity of fructokinase in the back reaction was measured by coupling the fructose produced with sorbitol dehydrogenase. Each assay mixture contained 20 mM fructose-1-P, 0.2 mM NADH, and 85 units of sorbitol dehydrogenase at pH 8.

*Data Analysis.* For fixed time assays, initial velocities were determined by least-squares analysis of the four time points. In all cases, reciprocal velocities were plotted graphically against reciprocals of substrate concentrations. The data were fitted to eq 2 with the Fortran program of Cleland (1967), assuming equal variance for the velocities.

$$v = VA/(K + A) \quad (2)$$

## Results

*Reaction with ATPβS.* Figure 1 shows the predominant complexes present when $Mg^{2+}$ or $Cd^{2+}$ ions were used with the $R_P$ or $S_P$ complexes of ATPβS and the relative $V/K$ values observed, while Table I includes the full kinetic data and comparative values for ATP complexes. A clear reversal in isomeric specificity when $Cd^{2+}$ replaces $Mg^{2+}$ is seen in both $V$ and $V/K$ values, so the Δ screw sense isomer is the substrate

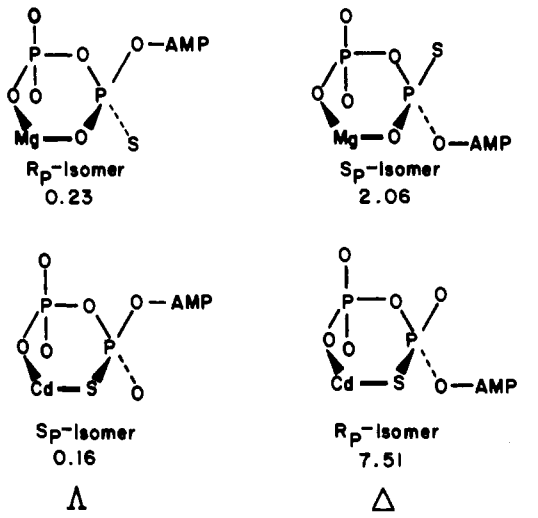


FIGURE 1: Thermodynamically preferred complexes of $Mg^{2+}$ and $Cd^{2+}$ with ATPBS isomers and the relative $V/K$ values observed in the present study.

for fructokinase. Note from Table I that CdATP is nearly as good a substrate for fructokinase as is MgATP. Free $Cd^{2+}$ is often inhibitory to enzymes, but with the total level being kept at 90% of the nucleotide level, this clearly does not occur. Inhibition was observed with CdATP when dithiothreitol was present at mM levels, but we believe this results from competition between ATP and dithiothreitol for $Cd^{2+}$ (comparison of the stability constants of other ligands with two sulfur atoms with that of CdATP suggests that a substantial portion of $Cd^{2+}$ would be present as a complex with dithiothreitol). Clearly dithiothreitol should not be present in appreciable levels when soft metal ions such as $Cd^{2+}$ are used to complex ATP.

Although a clear reversal in specificity is seen in Table I, more activity was observed with ($R_P$)-MgATPβS or ($S_P$)-CdATPβS than one would expect on the basis of the calculated level of Δ isomer present. Thus, the ratio of $V/K$ values for the $S_P$ and $R_P$ isomers of MgATPβS is only 9, while the expected ratio, based on the Δ screw sense content estimated by Pecoraro et al. (1984) as one part in 31 000 in the $R_P$ isomer and the effect of coordinated sulfur on the $V/K$ value of this isomer (see Table III), is ~6400. Similarly, the ratio of $V/K$ values for the $R_P$ and $S_P$ isomers of CdATPβS is 47, while the calculated ratio is 290. These data thus suggest either that the thionucleotides are not chirally pure or that they contain ATP. We believe the latter explanation is more likely and that the contamination occurred while changing the salt form with Dowex-50-$H^+$. We can calculate the degree of contamination as follows. When there are two active substrates and an inhibitor present, the rate equation for the reaction is

$$v = \frac{(V_a/K_a)A + (V_b/K_b)B}{1 + I/K_i + A/K_a + B/K_b} \quad (3)$$

where $A$ and $B$ are the concentrations of the active substrates,

Table II: Kinetic Constants for Reaction of ADPαS[a]

| metal ion | substrate | screw[b] sense | relative $V$ | relative[c] $V/K$ | $K_m$ (mM) |
|---|---|---|---|---|---|
| Mg | ADP | | | | 0.10[d] |
| Mg | ($S_P$)-ADPαS | Λ | (100) | 188 ± 8 | 0.53 ± 0.04 |
| Mg | ($R_P$)-ADPαS | Δ | 5.0 ± 0.5 | 2.2 ± 0.4 | 2.3 ± 0.6 |
| Cd | ($S_P$)-ADPαS | Δ | 14 ± 1 | 67 ± 19 | 0.21 ± 0.03 |
| Cd | ($R_P$)-ADPαS | Λ | 16 ± 2 | 27 ± 7 | 0.6 ± 0.2 |

[a] Data obtained at 25 °C, pH 8, with 20 mM fructose-1-P and 0.3 units/mL fructokinase. [b] Screw sense of the major coordination isomer present in solution. [c] Relative $V$ divided by $K_m$. [d] From Raushel & Cleland (1977) (pH 7).

$K_a$, $V_a$ and $K_b$, $V_b$ are the corresponding kinetic constants, $I$ is the inhibitor concentration, and $K_i$ is its dissociation constant. if $A$ is the MgATP contaminant, $B$ the minor Δ isomer of ($R_P$)-MgATPβS, and $I$ the major Λ isomer, we can write: $A = xI$ and $B = I/y$, where $y$ is expected to be 31 000 (Pecoraro et al., 1984). Substitution in eq 3 gives

$$v = \frac{I\,[(V_a/K_a)x + (V_b/K_b)/y]}{1 + I/K_i + Ix/K_a + Ix/(K_b y)} \qquad (4)$$

in which only the first numerator term[3] and the first two denominator terms are significant. The apparent $V/K$ value in terms of I (the major species present) is then $xV_a/K_a$, and since $V_a/K_a$ is simply the $V/K$ value for MgATP, we have from the values in Table I $197x = 0.23$, or $x = 1.17 \times 10^{-3}$. Thus, the $R_P$ isomer of ATPβS contained 0.12% ATP. We can then correct the values for ($R_P$)-CdATPβS in Table I by applying eq 3 with $A$ as CdATP and $B$ as the Δ isomer of ($R_P$)-CdATPβS, using the $V/K$ value for CdATP for $V_a/K_a$ and letting $A = xB$. This decreases the observed $V/K$ value from 7.51 to 7.43. The correction to $V$ is similar, since the level of contamination by ATP makes no appreciable difference to the denominator of eq 3 and thus to the apparent $K_m$ value.

A similar calculation for the $V/K$ values seen with the $S_P$ isomer of ATPβS gives 0.09% ATP as a contaminant. In this case both numerator terms in eq 4 are significant; about $^1/_6$ of the observed $V/K$ value is for the small level of Δ isomer of the thionucleotide complex, and $^5/_6$ is from CdATP.[4] The $V$ and $V/K$ values for ($S_P$)-MgATPβS can then be corrected for contamination by ATP to give the values shown in parentheses in Table I.

The $K_m$ values in Table I for the ATPβS complexes differ very little and average about 4 times higher than the values for MgATP or CdATP. The fact that the $K_m$ is almost unchanged when nearly all of the material is in the inactive Λ complex shows that nonproductive binding of the wrong screw sense isomer occurs with the same strength as productive binding of the correct one (the observed $K_m$ is in fact the dissociation constant of the inactive Λ complex in these cases). This lack of specificity may be contrasted with yeast hexokinase, where the correct screw sense isomer binds 5–10-fold more tightly than the incorrect one [Dunaway-Mariano & Cleland, 1980; see also the interpretation by Pecoraro et al. (1984) of the data of Jaffe & Cohn (1979)].

*Reaction with ADPαS.* Table II shows the activity of ADPαS isomers with fructose-1-P in the back-reaction with $Mg^{2+}$ and $Cd^{2+}$.

[3] The observed $V/K$ value for ($R_P$)-MgATPβS of 0.23 is almost entirely due to contaminating ATP. The estimated $V/K$ value resulting from activity of the minor Δ isomer is $(197/31\,000)/21 = 3 \times 10^{-4}$ (the value for MgATP divided by 31 000 to allow for the preferential binding of oxygen rather than sulfur to $Mg^{2+}$ and divided by 21 to allow for the effect of sulfur in the coordinated position; see Table III).

[4] The contribution from the Δ isomer is the $V/K$ value for CdATP (156) divided by 102 to allow for the effect of noncoordinated sulfur and by 60 to allow for the proportion of Δ isomer. The contribution from CdATP is $156x$, so that $x = (0.16 - 0.026)/156$.

Table III: Effect of Sulfur Substitution on the Kinetic Parameters for Fructokinase and Hexokinase

| | fructokinase[a] | hexokinase[b] |
|---|---|---|
| effect of noncoordinated sulfur on[c] | | |
| $V$ | ÷24 | ÷27.7 |
| $V/K$ | ÷102 | ÷74.8 |
| $K_m$ | ×4.22 | ×2.7 |
| effect of coordinated sulfur on[d] | | |
| $V$ | ÷6.2 | ÷5.09 |
| $V/K$ | ÷21 | ÷6.57 |
| $K_m$ | ×4.83 | ×1.29 |

[a] Calculated with values from Table I corrected for contamination with ATP. [b] Values calculated from the data of Jaffe & Cohn (1979). [c] The comparisons are between MgATP and ($S_P$)-MgATPβS. [d] The comparisons are between CdATP and ($R_P$)-CdATPβS.

## Discussion

*Stereochemistry at the β Position.* The data presented in this paper indicate that beef liver fructokinase utilizes the Δ isomer of MgATP as substrate. This preference is shared with phosphofructokinase, myokinase, and pyruvate kinase (Dunaway-Mariano & Cleland, 1980; Ngoc et al., 1979) but is the opposite of that observed for hexokinase and glycerokinase (Dunaway-Mariano & Cleland, 1980; Jaffe & Cohn, 1979), glucokinase (Darby & Trayer, 1982), and creatine kinase (Burgers & Eckstein, 1980). Since they also have the same substrate specificity for a β-fructofuranose structure, fructokinase and phosphofructokinase may be evolutionarily closely related, although the possible insertion of an enzyme group in the inner coordination sphere of the nucleotide-bound metal ion with fructokinase and not phosphofructokinase, for which CrATP is a substrate, is a difference.

In Table III we summarize the effects of sulfur in the coordinated and noncoordinated positions for both fructokinase and hexokinase, the only other enzyme for which a full kinetic analysis permits such calculations. The values in Table III are calculated by comparing MgATP or CdATP with the corresponding metal complexes of that ATPβS isomer in which the major screw sense form present is an active substrate. For both enzymes, the effects on $K_m$ are small compared to those on $V$ or $V/K$, but in all cases sulfur substitution decreases affinity somewhat. Although there are only two examples in Table II, the effects on $V$ are similar, and it is clear that substitution of sulfur in a noncoordinated position has a 4–5-fold greater effect on $V$ than does sulfur substitution in the coordinated position. The effects on $V/K$ show the same trend but are more variable. Note the high (up to 2 orders of magnitude) effect of noncoordinated sulfur on $V/K$; it is clear that contamination by ATP in ATPβS must be kept to 0.1% or less, or the observed reaction will simply be that of ATP!

The large decreases in $V$ and $V/K$ caused by sulfur substitution may partly result from increased bond lengths and thus distorted geometry, but the greater effect of substituion in the noncoordinated position of the β-phosphate suggests an electronic explanation, and indeed, this is likely to be the major cause of the effect. In MgATP or MgATPβS the noncoordinated oxygen or sulfur at the β position is largely double

bonded to phosphorus, and the coordinated atom is single bonded. After phosphate transfer, the original bridge oxygen and the noncoordinated $\beta$-oxygen become largely equivalent, and each will have $\sim$1.5 bond order to phosphorus. Thus, delocalization of electrons into the $\beta$ noncoordinated oxygen is occurring in the transition state. When the $\beta$ noncoordinated atom is sulfur, however, this delocalization is hindered, since noncoordinated sulfur prefers to be single bonded when possible (Frey & Sammons, 1985) [this is probably why the stability constant of MgATP$\beta$S is less than that of MgATP by more than the expected statistical factor of 2 (Pecoraro et al., 1984)]. The effect of sulfur substitution in the coordinated position may reflect a decreased transference via sulfur as opposed to oxygen of the electrophilic effect of the metal ion to phosphorus. Such explanations seem required, since the p$K$ of ADP$\beta$S (the leaving group in the transphosphorylation reaction) is actually lower than that of ADP, so that on this basis sulfur substitution in either position should speed up the reaction. The equilibrium constant for phosphate transfer is also more favorable by a factor of 60 with ATP$\beta$S than with ATP, which again would tend to make the reaction faster, not slower.

The calculations above demonstrate the importance of obtaining a full kinetic analysis when doing $Mg^{2+}$–$Cd^{2+}$ reversal studies with sulfur-substituted nucleotides. One must not depend solely on $V$ ratios for determining screw sense specificity because nonproductive binding of the inactive isomers may alter $V$ ratios. The correct parameter to compare is $V/K$, and it is necessary to compare $V/K$ with each metal ion with ATP as well as with both isomers of the thionucleotide. Analysis as we have done will then yield the screw sense specificity of the enzyme, the effect on $V$, $K_m$, and $V/K$ of sulfur substitution in coordinated and noncoordinated positions, and the extent of nonproductive binding of the inactive screw sense isomers.

*Stereochemistry at the $\alpha$ Position.* The data in Table II show that with either $Mg^{2+}$ or $Cd^{2+}$ the $V/K$ value is higher with the $S_P$ isomer of ADP$\alpha$S, although the ratio is 86 with $Mg^{2+}$ and only 2.5 with $Cd^{2+}$. This lack of reversal suggests that the $\alpha$-phosphate is not coordinated during chemical reaction on the enzyme but that the reaction works better with the $S_P$ isomer, possibly because of specific hydrogen-bonding interactions with the oxygen of the $\alpha$-phosphate of this isomer which are not possible when sulfur occupies this position. It is notable, however, that both isomers have similar $K_m$'s, with the $S_P$ isomer binding more tightly by 3-fold with $Cd^{2+}$ and 4-fold with $Mg^{2+}$ (the $S_P$ isomer of ADP$\alpha$S with $Mg^{2+}$ has a $K_m$ 5-fold higher than that with MgADP, however). This suggests that there is considerable binding of both screw sense isomers, and in fact, the normal reaction course may involve binding of one of the predominant bidentate isomers from solution, followed by rearrangement in the presence of fructose-1-P to give a $\beta$-monodentate structure prior to phosphate transfer.[5]

Why then are the $V_{max}$ values nearly the same for the two isomers of ADP$\alpha$S with $Cd^{2+}$? These reactions are slow, and possibly a common step other than transphosphorylation (cleavage of the Cd–S bond during conversion of bidentate to the active monodentate structure on the enzyme?) may be totally rate limiting.

**Registry No.** MgATP, 1476-84-2; CdATP, 72052-13-2; ($R_P$)-MgATP$\beta$S, 72052-08-5; ($R_P$)-CdATP$\beta$S, 72052-15-4; ($S_P$)-MgATP$\beta$S, 72052-07-4; ($S_P$)-CdATP$\beta$S, 72052-14-3; MgADP, 7384-99-8; ($S_P$)-MgADP$\alpha$S, 79189-53-0; ($R_P$)-MgADP$\alpha$S, 79189-52-9; ($S_P$)-CdADP$\alpha$S, 79189-48-3; ($R_P$)-CdADP$\alpha$S, 79190-77-5; fructose, 57-48-7; fructokinase, 9030-51-7; hexokinase, 9001-51-8.

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[5] An alternative is that the substrate is $\beta$-monodentate MgADP, which can be estimated from the stability constants of MgADP and MgAMP (Frey & Stuehr, 1972) to be 3–4% MgADP in solution. There would be much less monodentate material in solutions of CdADP$\alpha$S, however, because the sulfur elevates the stability constant by a factor of 23 (Pecoraro et al., 1984), so that the content of the monodentate species should be reduced by the same factor (that is, to less than 0.2%). Thus, it seems likely that bidentate complexes adsorbed on the enzyme can become monodentate and serve as substrates.