Nakamaya, K., & Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679–9698.
Nickbarg, E. B. (1988) Ph.D. Thesis, Harvard University, Cambridge, MA.
Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5948–5960.
Pickover, C. A., McKay, D. B., Engelman, D. M., & Steitz, T. A. (1979) *J. Biol. Chem. 254*, 11323–11329.
Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 311–321.
Putman, S. J., Coulson, A. F. W., Farley, I. R. T., Riddleston, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 301–310.
Racker, E. (1957) *Methods Enzymol. 3*, 293–296.
Raines, R. T., Sutton, E. L., Straus, D. R., Gilbert, W., & Knowles, J. R. (1986) *Biochemistry 25*, 7142–7154.
Reddy, E. P., Reynolds, R. K., Santos, E., & Barbacid, M. (1982) *Nature (London) 300*, 149–152.
Reinstein, J., Brune, M., & Wittinghofer, A. (1988) *Biochemistry 27*, 4712–4720.
Richard, J. P. (1984) *J. Am. Chem. Soc. 106*, 4926–4936.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167–339.
Rieder, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007–1010.
Rose, I. A. (1962) *Brookhaven Symp. Biol. 15*, 293–309.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Sayers, J. R., Schmidt, W., & Eckstein, F. (1988) *Nucleic Acids Res. 16*, 791–802.
Seeburg, P. H., Colby, W. W., Capon, D. H., Goeddel, D. V., & Levinson, A. D. (1984) *Naure (London) 312*, 71–75.
Tabin, C. J., Bradley, S. M., Bargmann, C. I., Weinberg, R. A., Papageorge, A. G., Scolnick, E. M., Dhar, R., Lowy, D. R., & Chang, E. H. (1982) *Nature (London) 300*, 143–149.
Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., & Wigler, M. (1982) *Nature (London) 300*, 762–765.
Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A., & Eckstein, F. (1985a) *Nucleic Acids Res. 13*, 8749–8764.
Taylor, J. W., Ott, J., & Eckstein, F. (1985b) *Nucleic Acids Res. 13*, 8764–8785.
Tong, L., de Vos, A. M., Milburn, M. V., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S. (1989) *Nature (London) 337*, 90–93.
Wierenga, R. K., Terpstra, P., & Hol, W. G. J. (1986) *J. Mol. Biol. 187*, 101–107.
Winn, S. I., Watson, H. C., Harkins, R. N., & Fothergill, L. A. (1981) *Philos. Trans. R. Soc. London, B 293*, 121–130.

Kinetic Competence of Enzymic Intermediates: Fact or Fiction?†

W. W. Cleland

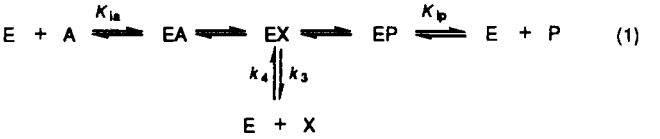
Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, 1710 University Avenue, Madison, Wisconsin 53705

Received October 2, 1989; Revised Manuscript Received December 14, 1989

ABSTRACT: A number of enzymatic reactions involve intermediates that are not normally released during the reaction. Whether such an intermediate when added to the enzyme reacts as fast or faster than the normal substrates, and thus is "kinetically competent", depends on the degree to which the equilibrium constant for forming the intermediate from the substrates is different on the enzyme surface and in solution, as well as on the relative affinities of the enzyme for substrate and intermediate. Similar values for these equilibrium constants require that the intermediate react slowly, while a far more favorable value for intermediate formation on the enzyme allows the intermediate to react at up to the diffusion-limiting rate. When one intermediate is formed from two substrates, it may react much more rapidly than when two intermediates are formed from two substrates, or one from one. Comparison of the kinetics of the putative intermediate(s) and the substrate(s) can reveal a great deal about the mechanism of the catalytic reaction and the kinetic barrier that normally keeps the intermediate(s) on the enzyme.

Many enzyme-catalyzed reactions involve multiple steps and intermediates that do not dissociate from the enzyme during the reaction. Such putative intermediates are in some cases stable enough to be prepared and tested as alternate substrates, and the question then arises how fast should such molecules react? One view is that in order for the intermediate to be "kinetically competent" it must react as fast or faster than the overall reaction of the substrate or substrates that it replaces. An alternative view is that because the "off" rate constant for an intermediate that does not normally dissociate is very small, the "on" rate constant must also be small, or the intermediate will be so tightly bound that it would exist in a deep hole in the free-energy profile for the reaction and its reaction in either direction would be too slow. Which view is correct?

It turns out that both situations are possible, with the controlling factor being the ratio of equilibrium constants between substrate and intermediate on and off the enzyme. Consider the simple mechanism



(1)

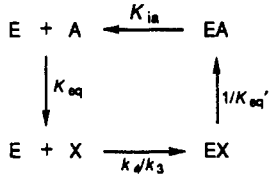
where A and P are the normal substrate and product and X is the intermediate. K_{ia} and K_{ip} are the dissociation constants of A from EA and P from EP. X does not normally dissociate

†Supported by NIH Grant GM 18938.

0006-2960/90/0429-3194$02.50/0

($k_3 < 10^{-3}$ s^{-1}), and the question is what value can k_4 have?

We can construct a thermodynamic box:



(2)

where we show the equilibrium constants for each step in the direction of the arrows. K_{eq} and K_{eq}' are the equilibrium constants for conversion of A to X off and on the enzyme, respectively. Since the overall equilibrium constant for the steps around such a box must be unity, we have

$$K_{eq}k_4K_{ia}/K_{eq}'k_3 = 1.0 \quad (3)$$

or

$$k_4 = k_3K_{eq}'/K_{ia}K_{eq} \quad (4)$$

The value of k_4 in M^{-1} s^{-1} is thus determined not only by how tightly the substrate binds to the enzyme (K_{ia} in molar units) but also by the ratio of the equilibrium constants for conversion of substrate to intermediate on and off the enzyme (K_{eq}'/K_{eq}).

Consider fumarase, which interconverts malate and fumarate via a carbanion intermediate (Blanchard & Cleland, 1980). The formation of the carbanion from malate is facile and comes to equilibrium on the enzyme at neutral pH, with C–O bond breaking and release of fumarate being the rate-limiting steps (Blanchard & Cleland, 1980). K_{eq}' is thus not far from unity, and this is the general situation for enzymes (Albery & Knowles, 1976). K_{eq}, however, is less than 10^{-13} at pH 7, since the pK of the CH_2 group of malate certainly exceeds 20. Since K_{ia} is 10^{-4} M, $k_4 > 10^{17}k_3$, and it is clear that, even if k_4 were diffusion limited ($\sim 10^9$ M^{-1} s^{-1}), k_3 would be less than 10^{-8} s^{-1}. Thus, the very forces that the enzyme brings to bear to stabilize the carbanion intermediate prevent its dissociation from the enzyme. There is no thermodynamic reason, however, why the carbanion (if it *were* stable in solution) could not react with fumarase in a diffusion-limited fashion, and thus appear kinetically competent.

Consider what would happen, however, if K_{eq} and K_{eq}' were the same size. In this case (with K_{ia} still equal to 10^{-4} M), k_4 is 10^4k_3, and if k_3 is less than 10^{-3} s^{-1}, k_4 is less than 10 M^{-1} s^{-1}. In this case the intermediate would appear *not* to be kinetically competent.

The model we have used, however, is an oversimplified one, since we have assumed that EX forms directly from E and X. A more realistic mechanism would be

$$EX \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EX' \overset{K_{ix}}{\rightleftharpoons} E + X \quad (5)$$

where both k_3 and k_4 are first-order rate constants and K_{ix} is the dissociation constant of the intermediate from the collision complex EX′. We presume that k_3 is again small ($<10^{-3}$ s^{-1}), so that X does not dissociate. Equation 4 now becomes

$$k_4 = k_3K_{eq}'K_{ix}/K_{ia}K_{eq} \quad (6)$$

and it is clear that if $K_{eq}' \simeq K_{eq}$ and $K_{ix} \simeq K_{ia}$, then $k_4 \simeq k_3$, and thus the intermediate will appear not to be kinetically competent. As the ratio of K_{eq}' to K_{eq} increases, however, the potential value of k_4 increases, so that it may no longer be rate limiting if K_{eq}'/K_{eq} has a high enough value. The value of k_4 could also be much greater than k_3 if $K_{eq}' \simeq K_{eq}$, while $K_{ix} \gg K_{ia}$. However, this is an unlikely situation, and K_{ix} is more likely to be less than, rather that greater than, K_{ia}.

If there are two or more substrates, the equations are similar, except that the dissociation constants of all substrates are in the denominators of eqs 4 and 6. Likewise if there are two or more intermediates present at once on the enzyme, all of their dissociation constants will appear in the numerator of eq 6. It will still be the ratio of K_{eq}'/K_{eq} that largely determines the ratio of k_4 to k_3, unless the affinities of the enzyme for intermediate and substrate are greatly different or unless the number of intermediates is less than the number of substrates (see below).

Let us analyze several examples of the activity of putative reaction intermediates. The first is formyltetrahydrofolate synthetase, which catalyzes reaction 7 in which the intermediates do not normally dissociate. Formyl phosphate has been shown to combine with the enzyme in the presence of MgADP and THF and to partition to give both formate and formyl-THF as products in roughly the expected ratio (Smithers et al., 1987). The K_m for formyl phosphate was 3 mM, but the turnover number was only 5 s^{-1} for formation of formyl-THF, compared to a value of 150 s^{-1} for reaction of formate, THF, and MgATP to give the same product. Thus at first glance formyl phosphate does appears not to be kinetically competent. However, K_{eq} for formation of formyl phosphate from MgATP and formate will be about 3×10^{-4}, while on the enzyme K_{eq}' is thought to be $\sim$4.3 (Mejillano et al., 1989). Equation 6 thus becomes

$$\text{MgATP + formate + THF} \rightleftharpoons \text{[E·formyl-P·MgADP·THF]} \rightleftharpoons \text{formyl-THF + P}_i\text{ + MgADP} \quad (7)$$

$$k_4 = K_{eq}'k_3K_{ix}K_{iq}K_{ic}'/K_{eq}K_{ia}K_{ib}K_{ic} \quad (8)$$

In eq 8, K_{ia}, K_{ib}, and K_{ic} are the dissociation constants of formate, MgATP, and THF for the normal forward reaction [8.3, 0.22, and 0.37 mM, respectively (Himes & Harmony, 1973)], while K_{ix}, K_{iq}, and K_{ic}' are the dissociation constants of formyl phosphate, MgADP, and THF for the reaction with formyl-P as substrate [3.4, 0.04, and 0.25 mM, respectively (Smithers et al., 1987)]. Assuming these values, k_4 will be $730k_3$. If the turnover number of 5 s^{-1} reflects the value of k_4, then k_3 would have to be 0.007 s^{-1}, so that formyl phosphate might dissociate from the enzyme once in every 22 000 turnovers (from comparison of 0.007 with the turnover number of 150 s^{-1}). Thus, the lack of "kinetic competence" does not show that formyl phosphate is not an intermediate but merely reflects the fact that K_{eq} is not that much smaller than K_{eq}', while k_3 has been made small enough for formyl phosphate to stay bound to the enzyme.

This enzyme, in fact, acts as a very slow formate kinase in the presence of MgATP and formate, but in the absence of THF. The K_m's of formate and MgATP (2.2 and 0.42 mM) are similar to those of the overall reaction, while the forward and reverse rate constants for phosphorylation of formate on the enzyme were estimated as 5.2 and 1.2 s^{-1} (Mejillano et al., 1989). The rate constant for release of formyl phosphate was 0.03 s^{-1}, and the turnover number was 0.024 s^{-1} for the kinase activity. Formyl phosphate is released $\sim$4 times faster than we have calculated above; either the presence of THF decreases this rate constant, or the difference reflects error in our calculations as the result of using data from different experiments.

A second example is EPSP synthase, which catalyzes the reaction

$$E + S\text{-}3\text{-}P + PEP \rightleftharpoons [E{\cdot}X] \rightleftharpoons E + P_i + EPSP \quad (9)$$

where S-3-P, EPSP, and X are shikimate 3-phosphate, 5-en-

olpyruvylshikimate 3-phosphate, and the 2-phospho-5-shikimate 3-phosphate 2-hemiketal. The intermediate can be isolated by rapid denaturation by neat triethylamine of the enzyme at equilibrium with its reactants and is 30% of the material bound to the enzyme at equilibrium (Anderson et al., 1988a). Thus, K_{eq}' is not far from unity [a value of 12 was estimated from rapid-quench studies (Anderson et al., 1988b)]. The intermediate recombines with the enzyme and partitions to the normal products with a rate constant of 5×10^7 M^{-1} s^{-1} (Anderson & Johnson, 1989), and it thus appears kinetically competent.

We can rearrange the equation analogous to eq 4 when there are two substrates and one intermediate to

$$k_3 = k_4 K_{ia} K_{ib} K_{eq}/K_{eq}' \quad (10)$$

where K_{ia} and K_{ib} are the dissociation constants of shikimate 3-phosphate (7 μM) and PEP (18 μM), K_{eq}' is 12, and k_4 is 5×10^7. The value of K_{eq} can be approximated by breaking down the reaction of PEP and S-3-P into the following steps: (1) hydrolysis of PEP to enolpyruvate ($K_{eq} = 1.8 \times 10^4$ M; calculated from the free energy of hydrolysis of PEP and the degree of enolization of pyruvate); (2) ketonization of pyruvate [$K_{eq} = 2.5 \times 10^5$ (Burgner & Ray, 1974)]; (3) hemiketal formation between pyruvate and S-3-P; (4) phosphorylation of the hemiketal. If hemiketal formation has the same equilibrium constant as hydration of pyruvate [1.0×10^{-3} M^{-1}, if the concentration of water is considered (Pocker et al., 1969)] and the free energy of hydrolysis of the phosphorylated hemiketal is the same as that of glucose 1-phosphate, or of PEP to the enol, which have about the same values, then the overall K_{eq} value in solution is $(1.8 \times 10^4)(2.5 \times 10^5)(1 \times 10^{-3})/(1.8 \times 10^4) = 250$ M^{-1}. If hemiketal formation is less favored than hydration by an order of magnitude, K_{eq} would be 25 M^{-1}.

The enzyme makes the reaction of shikimate 3-phosphate and PEP unimolecular by adsorbing both substrates, but the value of K_{eq}' of 12 corresponds to the ratio of intermediate to reactant that would be seen if the concentration of the other reactant were 48 mM and K_{eq} were 250 M^{-1}. If K_{eq} were 25 M^{-1}, the other reactant concentration would have to be 480 mM to give the same degree of reaction. Thus the enzyme does not shift the equilibrium toward the intermediate very strongly, presumably in order to avoid having it too stable to react further. The enzyme does, of course, greatly accelerate the rate of formation and breakdown of the intermediate.

If K_{eq} is taken as 250 M^{-1}, eq 10 gives $k_3 = 0.13$ s^{-1}, and comparison of k_3 and k_4 gives a dissociation constant for the intermediate of 2.6 nM. If K_{eq} is 25 M^{-1}, $k_3 = 0.013$ s^{-1}, and the dissociation constant is 0.26 nM. These values for k_3 are high for an intermediate that one does not expect to dissociate, but the low dissociation constant will ensure that no appreciable concentration of intermediate builds up in solution (that is, most molecules that dissociate will recombine). The rate of spontaneous decomposition of the isolated intermediate is 2×10^{-4} s^{-1} at pH 7 (Anderson & Johnson, 1989), which is so low that even if 2.6 nM intermediate existed in equilibrium with the enzyme, its rate of breakdown would only be 5×10^{-7} μM s^{-1}, which is trivial compared to the K_m levels of the substrates.

The reason the intermediate in this reaction can react so rapidly with the enzyme and appear kinetically competent, despite the minimal change in equilibrium constant on the enzyme, is that two substrates, both of which have low dissociation constants, react together to form a single intermediate. This can be seen by comparing eq 10 with eq 4. The extra K_{ib} term in eq 10 with a value $\sim 10^{-5}$ M more than overcomes the ratio of K_{eq}/K_{eq}' of 2 or 20 M^{-1} (depending which value of K_{eq} we use) and permits k_4 to be larger by 4–5 orders of magnitude than it would be if the number of intermediates equaled the number of substrates, as in the formyl-THF synthetase case.

In a reaction analogous to EPSP synthase, ribulosebisphosphate carboxylase catalyzes the formation of two molecules of 3-phosphoglycerate from ribulose 1,5-bisphosphate and CO_2 via an intermediate that appears to be 2-carboxy-3-keto-D-arabinitol 1,5-bisphosphate (CKABP) (Pierce et al., 1986):

$$E + \text{ribulose bis-P} + CO_2 \rightleftharpoons [E\cdot CKABP] \rightarrow 2(\text{3-P-glycerate}) \quad (11)$$

The CKABP intermediate can be released from the enzyme by an acid quench and is released as the hydrate, since only 50% of ^{18}O in the original ketone is found after borohydride reduction of the released CKABP (Lorimer et al., 1986). CKABP exists almost entirely as the ketone in solution, however, and NMR failed to detect any hydrate [<7% (Pierce et al., 1986)]. CKABP is a slow substrate for the activated enzyme, being converted to 2 mol of 3-phosphoglycerate at a rate of 0.06–0.08 s^{-1}, which a K_m of 1.5 μM (Pierce et al., 1986). This is considerably slower than the normal turnover number of 3 s^{-1} and raises the question of kinetic competence. Equation 6 for this situation is

$$k_4 = k_3 K_{eq}' K_{ix}/K_{eq} K_{ia} K_{ib} \quad (12)$$

where K_{ia}, K_{ib}, and K_{ix} are dissociation constants of ribulose bisphosphate, CO_2, and CKABP. Taking K_{ia} and K_{ib} as the Michaelis constants (90 and 14 μM) may not be completely valid, especially for CO_2 which may react chemically with the enolate of ribulose bisphosphate without prior complex formation. But if we do so and assume K_{eq}' to be unity, we get

$$k_4 = 1200 k_3/K_{eq} \quad (13)$$

where K_{eq} and 1200 both have units of M^{-1}. Since CKABP does not normally dissociate, k_3 must be less than 10^{-3} s^{-1}, and K_{eq} will be 10^{-3} M^{-1} or less. Thus, there is no reason why k_4 should not be fast enough to exceed the normal turnover rate of 3 s^{-1}, unless the value of k_3 is very small. If this model is correct, the observed rate of ~ 0.07 s^{-1} for k_4 requires a k_3 value of $\sim 6 \times 10^{-8}$ s^{-1}, which is so slow that we are led to suspect that CKABP may not be kinetically competent.

The reason that CKABP may not be kinetically competent is the likely possibility that the free ketone is never an enzyme-bound intermediate in the normal reaction. The chemistry of the reaction may involve attack of water at C-3 on one face of the enediol with electrons from the double bond attacking CO_2 at C-2 on the opposite face of the enediol. This concerted reaction would produce the hydrated form of CKABP directly, and the deprotonation of one of the hydroxyl groups at C-3 would then result in C–C cleavage and formation of two 3-phosphoglycerate molecules. The observed turnover number of ~ 0.07 s^{-1} with CKABP is presumably the rate of ketone hydration on the enzyme. This is ~ 30 times faster than hydration in solution [2.5×10^{-3} s^{-1}, measured from the rate of ^{18}O exchange (Lorimer et al., 1986)], but the enzyme normally catalyzes attack of water on C-3 when this carbon is trigonal and thus has a general base in the correct position to accept a proton from water during the reaction. The attack of water is faster when the double bond is between C-2 and C-3 (>3 s^{-1}) than when it is in the keto group, and this likely results from the lack of general-acid assistance in the latter case, since in the enediol intermediate the 3-hydroxyl group present still carries the proton it has in ribulose bis-

phosphate and no general-acid catalysis is necessary.

Malic enzyme catalyzes the stepwise oxidative decarboxylation of L-malate by TPN to give TPNH, pyruvate, and CO_2, with dehydrogenation preceding decarboxylation, so that an intermedate E·TPNH·oxalacetate complex exists (Hermes et al., 1982):

$$E + TPN + malate \rightleftharpoons [E\cdot TPNH\cdot OAA] \rightleftharpoons E + TPNH + pyruvate + CO_2 \quad (14)$$

Oxalacetate does not dissociate during the reaction, but if added to E·TPNH, it partitions to both pyruvate and malate in a ratio of 0.47 (Grissom & Cleland, 1985) and with a total rate (of $\sim 1\ s^{-1}$) that is about 3% that of the forward reaction and 10% that of the normal reductive carboxylation of pyruvate to malate (Tang & Hsu, 1973). In this case, eq 6 becomes

$$k_4 = k_3 K_{eq}' K_{i\,TPNH} K_{i\,OAA} / K_{eq} K_{i\,TPN} K_{i\,malate} \quad (15)$$

K_{eq} is 2×10^{-6} at pH 7, and we will assume K_{eq}' is unity (the exact value is not known). $K_{i\,TPNH}$, $K_{i\,OAA}$, $K_{i\,TPN}$, and $K_{i\,malate}$ are 0.83 μM, 10 mM, 5.9 μM, and 1.6 mM, respectively (Schimerlik & Cleland, 1977), and if k_4 is $1\ s^{-1}$, we have

$$k_3 = 2.3 \times 10^{-6}\ s^{-1} \quad (16)$$

This is a reasonable, if low, value, and we can conclude that oxalacetate reacts slowly with E·TPNH because of the kinetic barrier. Note that if k_3 were 100-fold higher, oxalacetate would still not be released from the enzyme at an appreciable rate, but k_4 would now be $100\ s^{-1}$, which exceeds the forward turnover rate and is 10 times the turnover rate in the reverse direction. Clearly some enzymes provide higher kinetic barriers for dissociation of intermediates than others, and analysis of the apparent kinetic competence of such intermediates permits evaluation of the actual kinetic barrier.

A number of other potential intermediates can be tested for kinetic competence. For example, acyl adenylates are thought to be intermediates in the formation of aminoacyl-tRNAs, acyl-CoA thioesters, and a number of similar reactions. Acyl adenylates were shown in the 1960s and 1970s to act as alternate substrates for several of these enzymes, and they usually reacted at rates comparable to the overall reactions. We have not found detailed kinetic data similar to those used in the examples discussed above, however, so a similar analysis is not possible at this time. The theory developed here could clearly be applied to these cases, as well as to others, and should permit a more realistic evaluation of the "kinetic competence" of intermediates in enzymatic reactions.

References

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631.

Anderson, K. S, & Johnson, K. A. (1990) *J. Biol. Chem.* (in press).

Anderson, K. S., Sikorski, J. A., Benesi, A. J., & Johnson, K. A. (1988a) *J. Am. Chem. Soc. 110*, 6577.

Anderson, K. S., Sikorski, J. A., & Johnson, K. A. (1988b) *Biochemistry 27*, 7395.

Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4506.

Burgner, J. W., II, & Ray, W. J., Jr. (1974) *Biochemistry 13*, 4229.

Grissom, C. B., & Cleland, W. W. (1985) *Biochemistry 24*, 944.

Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.

Himes, R. H., & Harmony, J. A. K. (1973) *CRC Crit. Rev. Biochem. 1*, 501.

Lorimer, G. H., Andrews, T. J., Pierce, J., & Schloss, J. V. (1986) *Philos. Trans. R. Soc. London, B 313*, 397.

Mejillano, M. R., Jahansouz, H., Matsumaga, T. O., Kenyon, G. L., & Himes, R. H. (1989) *Biochemistry 28*, 5136.

Pierce, J., Andrews, T. J., & Lorimer, G. H. (1986) *J. Biol. Chem. 261*, 10248.

Pocker, Y., Meany, J. E., Nist, B. J., & Zadorojny, C. (1969) *J. Phys. Chem. 73*, 2879.

Schimerlik, M. I., & Cleland, W. W. (1977) *Biochemistry 16*, 565.

Smithers, G. W., Jahansouz, H., Kofron, J. L., Himes, R. H., & Reed, G. H. (1987) *Biochemistry 26*, 3943.

Tang, C. L., & Hsu, R. Y. (1973) *Biochem. J. 135*, 287.