Biochemistry

 Volume 38, Number 20 May 18, 1999

Accelerated Publications

Remarkable Stabilization of Zwitterionic Intermediates May Account for a Billion-fold Rate Acceleration by Thiamin Diphosphate-Dependent Decarboxylases†

Frank Jordan,* Haijuan Li, and Angela Brown

Departments of Chemistry and Biological Sciences and Program in Cellular and Molecular Biodynamics, Rutgers, the State University, Newark, New Jersey 07102

Received February 16, 1999

ABSTRACT: When the E91D variant of apo-yeast pyruvate decarboxylase (EC 4.1.1.1) is exposed to C2α-hydroxybenzylthiamin diphosphate, this putative intermediate is partitioned on the enzyme between release of the benzaldehyde product (as evidenced by regeneration of active enzyme) and dissociation of the proton at C2α to form the enamine−C2α-carbanion intermediate. While the pK_a (the negative log of the acid dissociation constant) for this dissociation is ∼15.4 in water, formation of the enamine at pH 6.0 on the enzyme indicates a >9 unit pK_a suppression by the enzyme environment. The dramatic stabilization of this zwitterionic enamine intermediate at the active center is sufficient to account for as much as a 10^9-fold rate acceleration on the enzyme. This "solvent" effect could be useful for achieving the bulk of the rate acceleration provided by the protein over and above that afforded by the coenzyme on all thiamin diphosphate-dependent 2-oxo acid decarboxylases.

Thiamin diphosphate (ThDP,[1] the vitamin B_1 coenzyme) is the cofactor responsible for enzymatic decarboxylations of 2-oxo acids (*1*). Its structure (*2*, *3*) and function on pyruvate decarboxylase (PDC, EC 4.1.1.1) are shown in Figure 1. The accepted mechanism involves the intermediacy in the reaction of two unstable zwitterionic intermediates: the C2-carbanion/ylide/carbene and the C2α-carbanion or enamine. The mechanism also invokes three covalent ThDP-bound intermediates: the C2α-lactylThDP (LThDP, a ThDP−substrate adduct), C2α-hydroxyethylidene-ThDP (the enamine produced by the decarboxylation), and the C2α-hydroxyethylThDP (HEThDP, a ThDP−product adduct). In solution, the formation of these unstable zwitterionic intermediates is evidenced by their high pK_as (pK_a is the negative log of the acid dissociation constant for dissociation of the positively charged stable species to the zwitterionic intermediates). In particular, the currently mentioned value for the pK_a at C2 is 17−19 in water (*4*); it is 15.4 at C2α for the C2α-hydroxybenzylthiazolium salt in water (*5*, *6*) and >15 for the C2α-hydroxyethylthiazolium salt in DMSO/water mixtures (*7*). In pure DMSO, the pK_a is 14 at C2α for the C2α-hydroxyethylthiazolium salt and 12.5 for the C2α-hydroxybenzylthiazolium salt analogue (*8*). The enamine intermediate has been characterized extensively in solution (*9*) and has also been observed on PDC when derived from highly conjugated pyruvate analogues (*10*−*12*). Until now, there has been no report on the determination of either pK_a on any ThDP enzyme. There is a report, however, that

† Supported by NIH Grant GM-50380, the Rutgers Busch Biomedical Fund, the Rutgers Board of Governors Fund, Hoffmann LaRoche Diagnostics Division (Somerville, NJ), and NSF Grant BIR94/13198.

* To whom correspondence should be addressed. Telephone: (973) 353-5470. Fax: (973) 353-1264. E-mail: frjordan@newark.rutgers.edu.

[1] Abbreviations: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase from *Saccharomyces cerevisiae* overexpressed in *Escherichia coli*; E91D PDC, variant with glutamate to aspartate substitution at position E91; LThDP, C2α-lactylThDP; enamine, C2α-hydroxyethylideneThDP; HEThDP, C2α-hydroxyethylThDP; HBThDP, C2α-hydroxybenzylThDP.

10.1021/bi990373g CCC: $18.00
Published on Web 04/29/1999

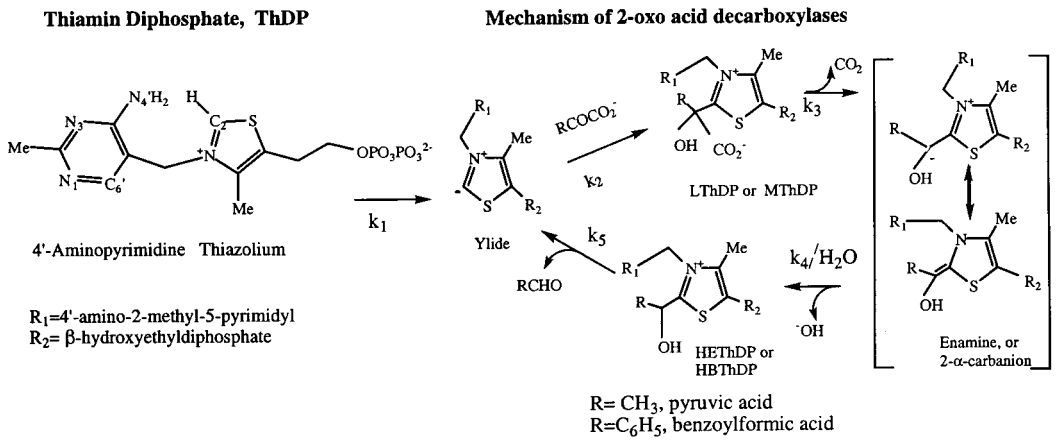

FIGURE 1

concluded (on the basis of ^{13}C NMR experiments) that on PDC, ThDP is undissociated at the C2 position (*13*). Given the difficulty of such experiments with an enzyme with the mass of PDC (240 kDa), as much as 20−30% in the conjugate base form could have been missed, due to the different relaxation characteristics of protonated and non-protonated carbon nuclei, and the fact that the chemical shift for C2 in the conjugate base zwitterionic form had only more recently been modeled (*14*, *15*).

We here present direct evidence for dramatic pK_a suppression at C2α of an intermediate resembling HEThDP on yeast PDC. The results could account for a very large fraction of the rate acceleration attributed to the protein in ThDP enzymes.

EXPERIMENTAL PROCEDURES

Construction of the E91D Variant of Saccharomyces cerevisiae PDC in Escherichia coli. Mutagenesis reactions were performed according to the PCR megaprimer method, which needs two flanking primers, one mutagenic primer, and two rounds of PCR. A pET22b(+) vector containing the pyruvate decarboxylase gene was used as the template in the PCR. In the first round of PCR, the oligonucleotide 5′-CCGCGAAATTAATACGACTCACTATA-3′ was used as a flanking primer, and the oligonucleotide 5′-AACAC-CGACGTG**ATC**AGCGTAAGAACC-3′ [the glutamic acid at position 91 was changed to aspartic acid (bold) and the *Afl*III restriction site for mutant screening (underlined) was eliminated] was used as a mutagenic primer. In the second round of PCR, 5′-GTTATGCTAGTTATTGCTCAGCGGT-3′ was used as a flanking primer, and the mutagenic DNA fragment (0.3 kb) produced by the first round was used as a second primer (called megaprimer), giving one 1.8 kb fragment containing the desired mutation. Both the products of the second PCR and the plasmid pET22b(+):*PDC1* were digested by *Xba*I−*Sac*I. The *Xba*I−*Sac*I-digested mutagenic *PDC1* fragment was ligated into the *Xba*I−*Sac*I-digested pET22b(+) fragment. The ligation mixture was transformed into *E. coli* DH5α competent cells. The mutation was screened by digestion with *Afl*III. The mutations were confirmed by DNA sequence analysis using the primer 5′-CCGCGAAATTAATACGACTCACTATA-3′. The mutated plasmids were transformed into *E. coli* strain BL21 (DE3) for protein expression. The ThDP could be separated from the PDC protein by DEAE anion-exchange chromatography at pH 6.10; hence, the E91D variant was purified as the apoenzyme. Full catalytic activity could be recovered for the variant enzyme on incubation with 10 mM $MgSO_4$ and 10 mM ThDP in 100 mM potassium phosphate (pH 6.0) for 30 min at 25 °C.

Enzyme purification, activity measurements, and related experimental protocols have been described previously (*16*).

C2α-Hydroxybenzylthiamin diphosphate (HBThDP) was synthesized according to the method of ref *17*. ThDP (5.0 g) was dissolved in water (10 mL), and the pH was adjusted to 8.0 with 3 N NaOH. Benzaldehyde (16 mL) dissolved in methanol (23 mL) was then added to the mixture. The pH_{app} was maintained at 8.0 with addition of dilute NaOH, and the solution was kept homogeneous with periodic addition of methanol. The solution was stirred under nitrogen for 3 h, after which the pH was adjusted to 2.5 with HCl. The mixture immediately separated into two layers; the organic layer was discarded, and the aqueous layer was evaporated to dryness. The residue was then washed several times with acetone to remove benzaldehyde and benzoic acid. The final product was recrystallized from water/ethanol: ^1H NMR (D_2O/DCl) δ 2.37 (s, 3H), 2.43 (s, 3H), 3.39 (2H), 4.30 (2H), 6.48 (C2α-H), 6.65 (C6′-H), 7.40 (phenyl-H).

Thiochrome diphosphate was synthesized according to the method of ref *18*. ThDP (2.0 g) was dissolved in 200 mL of anhydrous methanol. Iodine (1.1 g) was then added, and the mixture was stirred in a stoppered flask until the iodine was dissolved. Potassium carbonate (1.2 g) was then added, and the mixture was stirred until the color of iodine disappeared and the solution color turned bright yellow. The solution was then filtered and evaporated to give yellow crystals, which were recrystallized from ethanol: ^1H NMR (D_2O) δ 2.34 (3H), 2.58 (3H), 3.06 (2H), 4.16 (2H), 5.42 (2H), 8.03 (1H); ^{31}P NMR (D_2O) δ −2 (P-α), −14 (P-β).

Fluorescence Measurements. Fluorescence was recorded on a SLM 8100 spectrofluorimeter in a 0.8 mL quartz cuvette. The fluorescence intensity of the ternary complex, consisting of apo-PDC, Mg(II), and thiochrome diphosphate, is diminished by the addition of ThDP in a time- and concentration-dependent process (data not shown; see ref *19*). The emission spectrum of the complex with thiochrome diphosphate is distinct from the intrinsic PDC spectrum.

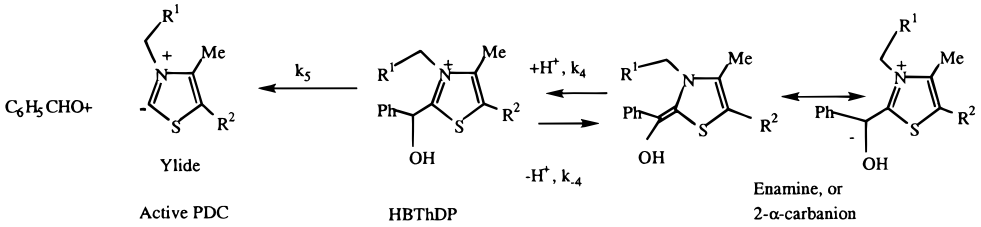

FIGURE 2: Proposed partitioning of HBThDP on pyruvate decarboxylase. The active enzyme results from the loss of benzaldehyde, while formation of the enamine intermediate is signaled by the observation of the characteristic absorbance at 380 nm.

RESULTS AND DISCUSSION

Experiments were designed to provide direct evidence for the pK_a at C2α of an intermediate resembling HEThDP on yeast PDC. The premise of the experiments is outlined below. (1) We need a PDC variant that has a lower affinity for ThDP than the wild-type enzyme to readily generate the stable apoenzyme. Of more than 12 positions substituted so far in our laboratory (*1*), the E91D variant is appropriate in this regard (*19*). It has a specific activity that is less than 4-fold smaller than that of the wild-type enzyme, and while the variant loses its ThDP readily during purification, holo-PDC can be reconstituted with very little loss in activity. Residue E91 is located at a domain interface forming a hydrogen bond to the main chain amide of W412. Any substitutions at this position lower the affinity for ThDP. According to measurements of the quenching of intrinsic PDC fluorescence, the E91D apo-PDC has an approximately 20-fold lower affinity for ThDP than the wild-type apo-PDC (*19*). (2) We need a substrate−ThDP or product−ThDP covalent adduct that can be used to reconstitute apo-PDC to holo-PDC, and one that also forms an enamine intermediate that is a strong chromophore in the visible spectrum, with a λ_{max} far from the protein and coenzyme absorptions. The enamine formed from decarboxylation of benzoylformic acid (whose ThDP−product adduct is C2α-hydroxybenzylThDP, HBThDP) has a λ_{max} of 380 nm (*5*). (3) Since we attempted to study the feasibility of the reverse reaction, instead of monitoring intermediate formation from the substrate−ThDP adduct in the forward reaction, we must try to generate it from the ThDP−product adduct. That such an adduct is a true intermediate is suggested by our previous elucidation of the reverse reaction, in which acetaldehyde added to PDC resulted in the formation of acetoin (*20*), clearly implying the intermediacy of HEThDP. We therefore selected C2α-hydroxybenzylThDP (HBThDP) to reconstitute the apoenzyme to achieve goal 2, with the expectation that reconstitution will be a slow process giving us optimal opportunity for observing the intermediate. According to Figure 2, we added HBThDP to E91D apo-PDC and looked for partitioning of the bound HBThDP between benzaldehyde release on one hand and enamine formation on the other.

The following experiments clearly show that the partitioning of HBThDP takes place on PDC. As seen in Figure 3, the activity of E91D apo-PDC toward pyruvate decarboxylation is regained with time upon addition of HBThDP, albeit at an approximately 2-fold slower rate than when ThDP is being used. This experiment confirms that the E91D apo-PDC can carry out the step depicted with rate constant k_5 in Figure 2. The time course of enamine formation at 380 nm is illustrated in Figure 4, where the buildup is occurring at a rate similar to that of the recovery of activity, providing

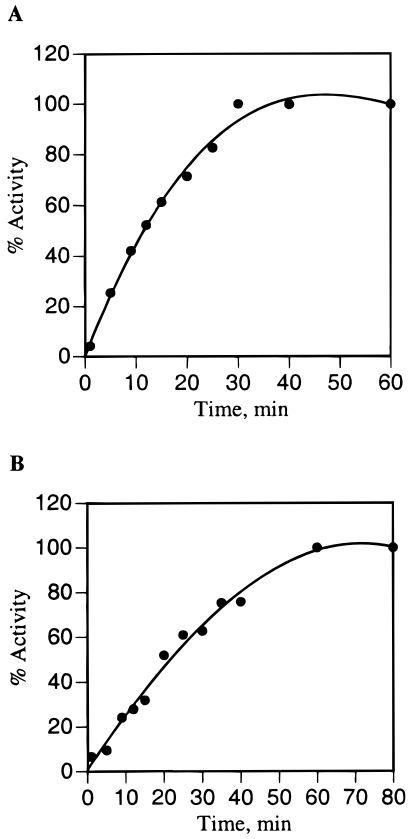

FIGURE 3: Time course of activation of the PDC apoenzyme with ThDP and HBThDP. E91D apo-PDC (0.5 mg/mL) was incubated with 20 mM $MgSO_4$ and 20 mM ThDP (A) or HBThDP (B) in 100 mM potassium phosphate (pH 6.10) at room temperature. At the indicated times, aliquots were removed and tested for pyruvate decarboxylating activity.

very strong evidence that we have induced the proton dissociation step depicted with rate constant k_{-4} in Figure 2. The lag seen in enamine formation could be due to a number of factors and is consistent with slower reactivation of apo-PDC with HBThDP than with ThDP. ThDP enzymes are subject to activation by ThDP·Mg(II), while yeast PDC is also subject to activation by substrate (*1*). There is no product in the reaction mixture other than the enamine that could account for the formation of a new chromophore. The decomposition of C2α-hydroxybenzylthiamin (reported by Kluger's group) takes place at a much slower rate than the reaction monitored here (*21*), and it leads to a product with a λ_{max} at 328 nm (ϵ = 10 000), in contrast to the spectral feature of the enamine produced here [λ_{max} at 380 nm (ϵ = 15 000)].

According to an estimate based on the extinction coefficient of a model for this enamine (*5*), the enzyme is fully saturated with the enamine; i.e., within experimental error,

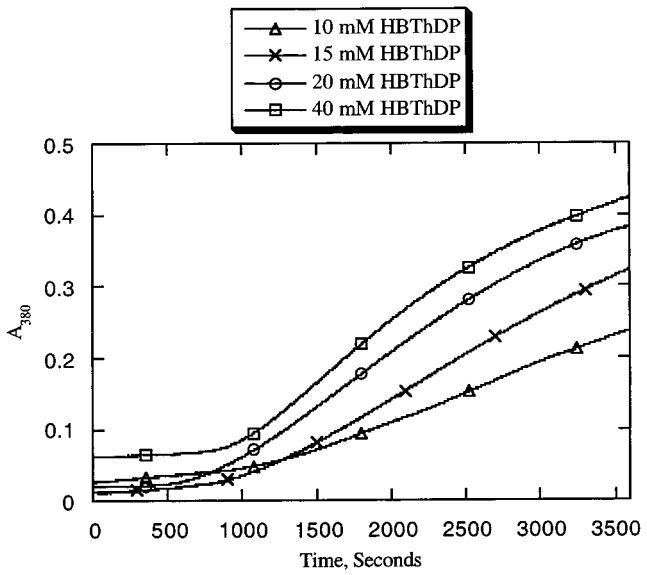

FIGURE 4: Formation of the enzyme-bound enamine derived from HBThDP. The E91D PDC apoenzyme (0.5 mg/mL) was incubated with 20 mM $MgSO_4$ in 100 mM potassium phosphate (pH 6.1) at room temperature, and then the indicated concentrations of HBThDP were added and the contents mixed immediately, followed by recording of the absorbance at 380 nm. The reference cuvette contained all components except the apoenzyme.

the HBThDP is fully dissociated at pH 6.0. We conclude that the pK_a at C2α of HBThDP bound to PDC is <6.0; i.e., it is lowered by more than 9 units by the enzyme, reflecting a >13 kcal/mol stabilization of the zwitterionic enamine intermediate on PDC.

We next asked whether the apparent dielectric constant at the active center could account for the pK_a suppression. For this purpose, we used a fluorescent ThDP analogue, thiochrome diphosphate. We first confirmed that thiochrome diphosphate is competitive with ThDP when it is bound to the wild-type apo-PDC (*22*). The fluorescence emission spectrum was measured for the ternary complex, consisting of apo-PDC, Mg(II), and thiochrome diphosphate. The emission maximum is between 422 and 424 nm (excitation at 290 nm). For calibration of the emission maximum against the solvent dielectric constant, we selected the series of 1-alkanols, from methanol to 1-hexanol. As can be seen in Figure 5, the emission maximum varies in a regular fashion with the dielectric constant, and the emission maximum observed with the enzyme falls between those of 1-pentanol and 1-hexanol; i.e., the apparent dielectric constant is 13−15. This apparent dielectric constant can be used to estimate the $\Delta\Delta G$ for transfer of an acid−base reaction (from a positively charged proton donor to a negatively charged acceptor) from water to a medium with a dielectric constant of 13−15. Depending on the distance between the proton donor and acceptor, $\Delta\Delta G$ varies from 5 kcal/mol for 4 Å to 6 kcal/mol for 3.2 Å (the distance between C2 and N4′; see ref *3*) and 7 kcal/mol for 2.6 Å (the heavy atom distance found in PDC in the three hydrogen bonds to the aminopyrimidine, all three conserved on all ThDP enzymes; see refs *2* and *3*). It can be concluded from this crude estimate that a medium with a low dielectric constant is nearly sufficient to account for the observed pK_a suppression. The presence of a nearby general acid−base catalyst for facilitating the proton transfer, such as D28, H115, or E477 (*1*, *23*), may

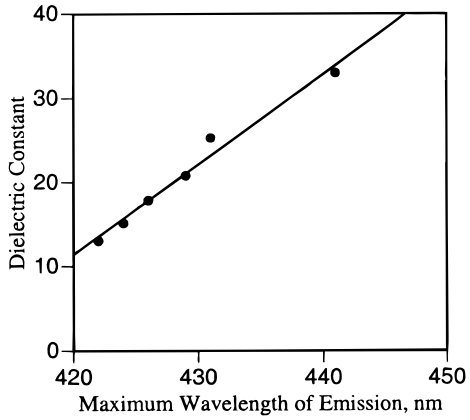

FIGURE 5: Plot of maximum fluorescence emission of thiochrome diphosphate in 1-alkanols with varying dielectric constants. The range is from methanol to 1-hexanol in the order of decreasing dielectric constant. In each solvent, the thiochrome diphosphate solution was excited at the wavelength maximum determined from the excitation spectrum and the fluorescence emission was recorded. The maximum in pH 6.0 buffer was 445 nm, and it was blue-shifted to 422−424 nm (excitation at 290 nm) when the species was bound to apo-PDC.

account for the remaining pK_a suppression.

The results also suggest that many earlier studies in which HEThDP has been used to study oxidative reactions of ThDP, such as with pyruvate oxidase and the pyruvate dehydrogenase and other 2-oxo acid dehydrogenase multienzyme complexes, do not measure the oxidation of this intermediate directly. Rather, the enzymes first induce dissociation of the C2αH, followed by oxidation at this carbon atom, as suggested by our model redox studies using electrochemical means (*24*), as well as our models for pyruvate oxidase (*25*) and for the 2-oxo acid dehydrogenase multienzyme complexes (*26*, *27*).

Is there a relationship between the magnitude of the observed pK_a suppression leading to the zwitterionic intermediates and the magnitude of the rate acceleration (related to the lowering of the activation barrier) of the steps leading to these zwitterionic intermediates? The pK_a suppression provides an upper limit to the achievable rate acceleration, and would be essentially equal to the barrier lowering for a reaction that follows Hammond's postulate for a "late" transition state (*28*). Paraphrased, this postulate states that transition states for strongly endothermic reactions, such as those leading to the very unstable intermediates discussed here, resemble the intermediates much more than the ground states of the reactions. The magnitude of the primary deuterium kinetic isotope effect (4−6) observed for this proton dissociation in model compounds (*6*) suggests, but does not prove, that this is likely to be the case on the enzyme as well. It has been estimated that PDC may accelerate the reaction by as much as 10^{12} compared to ThDP at pH 6.0 (*29*); thus, the >9 unit pK_a suppression may translate to a $>10^9$-fold rate acceleration, with the remaining contributions coming from the acid−base groups surrounding ThDP (*1*, *23*). It is to be further noted that while our results apply to stabilization of the C2α-enamine/zwitterion by PDC, one would expect the environment to produce a similar order of magnitude suppression of the pK_a of the thiazolium ring at C2H, as well as for ionization of the C2α-OH group likely to precede aldehyde release. The special "V" conformation

of the enzyme-bound ThDP, and its surroundings (*30*), may help to create this "solvent" effect. We further suggest that a similar enzymatic solvent effect is present on other ThDP-dependent 2-oxo acid decarboxylases for the same purpose, also accounting for a very large fraction of the observed rate acceleration.

The results confirm the intermediacy of intermediates such as HBThDP and the enamine suggested by Breslow's pioneering studies (*31*). The results also provide support for model studies by Lienhard's group, which had suggested the intervention of an important enzymatic solvent effect on ThDP enzymes, especially PDC (*32*). A simple inspection of the amino acid side chains at the active center per se cannot provide insight into the polarity of the active site (contrary to the deduction reached for PDC in ref *33*), while a fluorescence probe like that used here can indeed support or negate the existence of such a low-polarity environment. If stabilization of unstable intermediates on ThDP enzymes is, in large measure, indeed due to such solvent effects, there is no need to invoke any extraordinary acid−base chemistry, and particularly strong hydrogen bonds (perhaps of the low-barrier type), as suggested by a theoretical treatise for stabilization of unstable enols and enolates, somewhat related to the enamine in this study (*34*).

REFERENCES

1. Jordan, F., Nemeria, N., Guo, F., Baburina, I., Gao, Y., Kahyaoglu, A., Li, H., Wang, J., Yi, J., Guest, J. R., and Furey, W. (1998) *Biochim. Biophys. Acta 1385*, 287−306.
2. Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., and Jordan, F. (1993) *Biochemistry 32*, 6165−6170.
3. Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D., and Jordan, F. (1996) *J. Mol. Biol. 256*, 590−600.
4. Washabaugh, M. W., and Jencks, W. P. (1988) *Biochemistry 27*, 5044−5053.
5. Barletta, G., Huskey, W. P., and Jordan, F. (1992) *J. Am. Chem. Soc. 114*, 7607−7608.
6. Barletta, G., Huskey, W. P., and Jordan, F. (1997) *J. Am. Chem. Soc. 119*, 2356−2362.
7. Zou, Y. (1999) Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, Newark, NJ.
8. Bordwell, F. G., Satish, A. V., Rios, C. B., Chung, A., and Jordan, F. (1990) *J. Am. Chem. Soc. 112*, 792−797.
9. Jordan, F., Kudzin, Z. H., and Rios, C. B. (1987) *J. Am. Chem. Soc. 109*, 4415−4417.
10. Kuo, D. J., and Jordan, F. (1983) *J. Biol. Chem. 258*, 134l5−13417.
11. Zeng, X., Chung, A., Haran, M., and Jordan, F. (1991) *J. Am. Chem. Soc. 113*, 5842−5849.
12. Menon-Rudolph, S., Nishikawa, S., Zeng, X., and Jordan, F. (1992) *J. Am. Chem. Soc. 114*, 10110−10112.
13. Kern, D., Kern, G., Neef, H., Tittmann, K., Killenberg-Jabs, M., Wikner, C., Schneider, G., and Hübner, G. (1997) *Science 275*, 67−70.
14. Arduengo, A. J., Goerlich, J. R., and Marshall, W. J. (1997) *Liebigs Ann./Recl.*, 365−374.
15. Brown, A. (1999) Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, Newark, NJ.
16. Baburina, I., Li, H., Furey, W., Bennion, B., and Jordan, F. (1998) *Biochemistry 37*, 1235−1244.
17. Mieyal, J. J., Bantle, G., Votaw, R. G., Rosner, I. A., and Sable, H. Z. (1971) *J. Biol. Chem. 246*, 5213−5219.
18. Risinger, G. E., and Parker, P. N. (1965) *Experientia 21*, 305.
19. Li, H. (1999) Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, Newark, NJ.
20. Chen, G., and Jordan, F. (1984) *Biochemistry 23*, 3576−3582.
21. Kluger, R., Lam, J. F., Pezacki, J. P., and Yang, C.-M. (1995) *J. Am. Chem. Soc. 117*, 11383−11389.
22. Wittorf, J. H., and Gubler, C. J. (1970) *Eur. J. Biochem. 14*, 53−60.
23. Guo, F., Wang, J., and Jordan, F. Unpublished observations.
24. Barletta, G., Chung, A. C., Rios, C. B., Jordan, F., and Schlegel, J. M. (1990) *J. Am. Chem. Soc. 112*, 8144−8149.
25. Chiu, C. C., Pan, K., and Jordan, F. (1995) *J. Am. Chem. Soc. 117*, 7027−7028.
26. Chiu, C. C., Chung, A., Barletta, G., and Jordan, F. (1996) *J. Am. Chem. Soc. 118*, 11026−11029.
27. Pan, K., and Jordan, F. (1998) *Biochemistry 37*, 1357−1364.
28. Hammond, G. S. (1955) *J. Am. Chem. Soc. 77*, 334−338.
29. Alvarez, F. J., Ermer, J., Hübner, G., Schellenberger, A., and Schowen, R. L. (1991) *J. Am. Chem. Soc. 113*, 11026−11029.
30. Guo, F., Zhang, D., Kahyaoglou, A., Farid, R. S., and Jordan, F. (1998) *Biochemistry 37*, 13379−13391.
31. Breslow, R. (1957) *J. Am. Chem. Soc. 79*, 1762−1763.
32. Crosby, J., Stone, R., and Lienhard, G. E. (1970) *J. Am. Chem. Soc. 92*, 2891−2900.
33. Warshel, A. (1998) *J. Biol. Chem. 273*, 27035−27038.
34. Gerlt, J. A., and Gassman, P. G. (1993) *J. Am. Chem. Soc. 115*, 11552−11568.

BI990373G