Monomeric Sarcosine Oxidase: Evidence for an Ionizable Group in the E•S Complex[†]

Gouhua Zhao and Marilyn Schuman Jorns*

Department of Biochemistry, MCP Hahnemann School of Medicine, Philadelphia, Pennsylvania 19129

Received April 16, 2002; Revised Manuscript Received May 21, 2002

ABSTRACT: Monomeric sarcosine oxidase (MSOX) contains covalently bound FAD and catalyzes the oxidation of sarcosine (*N*-methylglycine) and other secondary amino acids, including L-proline. The reductive half-reaction with L-proline proceeds via a rapidly attained equilibrium (K_d) between free E_{ox} and the E_{ox}•S complex, followed by a practically irreversible reduction step (E_{ox}•S → E_{red}•P) associated with a rate constant, k_{lim}. The effect of pH on the reductive half-reaction shows that the K_d for L-proline binding is pH-independent (pH 6.46−9.0). This indicates that MSOX binds the zwitterionic form of L-proline, the predominant species in solution at neutral pH (pK_a = 10.6). Values for the limiting rate of reduction (k_{lim}) are, however, strongly pH-dependent and indicate that an ionizable group in the E_{ox}•L-proline complex (pK_a = 8.02) must be unprotonated for conversion to E_{red}•P. Charge-transfer interaction with L-proline as the donor and FAD as acceptor is possible only with the anionic form of L-proline. The ionizable group in the E_{ox}•L-proline complex is required for conversion of enzyme-bound L-proline from the zwitterionic to the reactive anionic form, as judged by the independently determined pK_a for charge-transfer complex formation with the MSOX flavin (pK_a = 7.94). The observation that the anionic form of L-proline with a neutral amino group is the reactive species in the reduction of MSOX is similar to that observed for other flavoenzymes that oxidize amines, including monoamine oxidase and trimethylamine dehydrogenase.

Monomeric sarcosine oxidase (MSOX) catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine), a common soil metabolite that can act as the sole source of carbon and energy for many microorganisms (*1*). This inducible bacterial enzyme contains 1 mol of FAD [8α-(S-cysteinyl)FAD] that is covalently linked to a cysteine residue (cys315) (*2*). The crystal structures of free MSOX and various enzyme•inhibitor complexes have been determined (*3*, *4*). MSOX is probably the best characterized member of a recently recognized family of prokaryotic and eukaryotic amine oxidoreductases, all of which contain covalently bound flavin (*2*, *5*, *6*).

The mechanism of flavoenzyme-catalyzed oxidation of amines remains highly controversial despite intensive study. The MSOX active-site structure and predicted substrate binding mode are consistent with at least four different mechanisms for amine oxidation, including two variants of a single electron transfer (SET) mechanism (*7*, *8*), a polar mechanism (*9*, *10*), and a hydride transfer mechanism (*11*) (Scheme 1). The two variants ofthe SET mechanism both involve initial one-electron transfer from substrate amino group to enzyme flavin, generating a flavin anion/substrate amine cation radical pair, but differ with respect to the mechanism of the second electron transfer. In one variant, proton loss from the substrate amine radical cation (pK_a ∼ 8−10) (*12*, *13*) yields a carbon-centered radical which then undergoes a second one-electron transfer to form the imine product and 1,5-dihydroFAD. In the other variant, the second electron is transferred from the substrate radical to the flavin radical as a hydrogen atom. The polar mechanism involves formation of a covalent flavin−substrate intermediate (4a−flavin adduct) and its subsequent rearrangement to yield 1,5-dihydroflavin plus imino acid. No intermediates are formed in the hydride transfer mechanism. Preliminary evidence in favor of a SET mechanism is provided by the observed inactivation of MSOX by (N-cyclopropyl)glycine (*14*).

The four mechanisms for sarcosine oxidation suggest three possible roles for an active-site base. (1) An unprotonated amino group is required in the two variants of the SET mechanism and the polar mechanism. Although not essential, an unprotonated amino group could promote hydride transfer by hyperconjugation. Sarcosine exists primarily as a zwitterion around neutral pH (pK_a = 2.33, 10.0). MSOX binds the zwitterionic form of dimethylglycine (an inhibitor) at pH 7, suggesting a similar binding mode for sarcosine (*4*). Conversion to the reactive anionic form may involve proton transfer to an active-site base. (2) In one variant of the SET mechanism, an active-site base might act as acceptor of the α-proton from the substrate amine radical cation. (3) The postulated rearrangement of the 4a−flavin adduct in the polar mechanism requires a base to remove the α-proton.

In this paper, we describe the effect of pH on the reductive half-reaction of MSOX with L-proline. The results show that a group with a pK_a = 8.0 in the E_{ox}•L-proline complex must

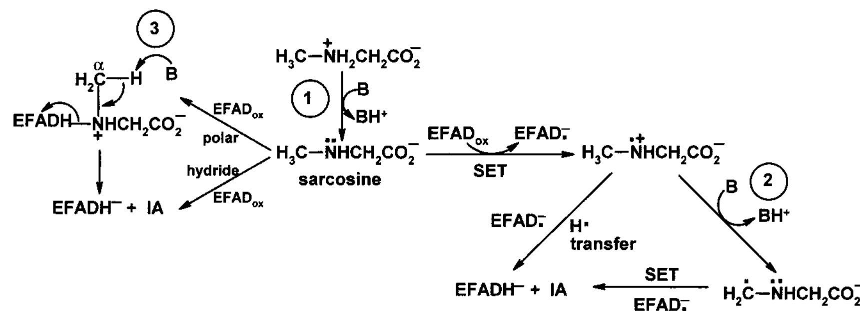
[†] This work was supported in part by Grant GM 31704 (M. S. J.) from the National Institutes of Health.

* Corresponding author. Phone: (215) 762-7495. Fax: (215) 762-4452. E-mail: marilyn.jorns@drexel.edu.

[1] Abbreviations: MSOX, monomeric sarcosine oxidase; FAD, flavin adenine dinucleotide; SET, single electron transfer.

10.1021/bi020285n CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/13/2002

Scheme 1: Four Possible Mechanisms for Sarcosine Oxidation by MSOX



be unprotonated for conversion to E_{red}•P. This group is also required for conversion of enzyme-bound L-proline from the zwitterionic to the reactive anionic form, as judged by the independently determined pK_a for charge-transfer complex formation with the MSOX flavin. Mutagenesis studies attempting to identify the ionizable group in the E•S complex are described in the accompanying manuscript.

EXPERIMENTAL PROCEDURES

Purification of MSOX. The recombinant enzyme was purified as previously described (*2*). During enzyme purification, MSOX activity was measured using the Nash assay, and protein was determined using the Bio-Rad micro protein assay (*2*).

Spectral Studies. Absorption spectra were recorded using a Perkin-Elmer Lambda 2S spectrometer. Anaerobic experiments were conducted using special cuvettes with two sidearms. The cuvettes were made anaerobic by purging with argon, as previously described (*4*).

Data Analysis. Data were fit to eqs 1−3 using the curve fit function in Sigma Plot (Jandel Corporation). Equation 1 was used for analysis of the apparent first-order rate of

$$Y = \frac{AX}{X + K} \qquad (1)$$

$$\log k_{lim} = \log(C)/(1 + [H^+]/K_a) \qquad (2)$$

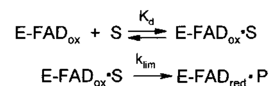
$$Y = \frac{AH^+ + BK_a}{H^+ + K_a} \qquad (3)$$

enzyme reduction by L-proline (k_{obs}) as a function of the substrate concentration. Y and A are k_{obs} and k_{lim}, respectively. X is the L-proline concentration, and K is the apparent dissociation constant of the E•S complex. Equation 2 was used to fit the reductive half-reaction kinetics with MSOX and L-proline as a function of pH. K_a is the ionization constant of a group in the E•S complex that must be unprotonated for enzyme reduction to occur. Equation 3 was used to fit the effect of pH on the absorption spectrum of the complex of MSOX with L-proline ($\lambda_{analysis}$ = 512 nm). Y is the observed absorbance change at 512 nm at a given pH value. A and B are the calculated absorbance change at this wavelength at low and high pH values, respectively.

RESULTS

Effect of pH on the Reductive Half-Reaction of MSOXwith L-Proline. L-Proline is a slow, alternate substrate for MSOX.

Scheme 2: Proposed Mechanism for MSOX Reduction by L-Proline



The reductive half-reaction is rate-limiting during turnover (*15*). We previously characterized the kinetics of the reductive half-reaction at pH 8.0 by monitoring the disappearance of oxidized flavin absorption at 454 nm. The reaction was found to exhibit apparent first-order kinetics over a wide range of L-proline concentrations (0.05−1.0 M). The observed apparent first-order rate constants exhibited a hyperbolic dependence on the concentration of L-proline. This result and other observations were found to be consistent with a mechanism where a rapidly attained equilibrium (K_d) between free E and the E•S complex is followed by a practically irreversible reduction step associated with a rate constant, k_{lim} (*15*) (Scheme 2).

The rate of the anaerobic half-reaction with L-proline is highly dependent on pH. Values for k_{lim} and K_d at different pH values were obtained by monitoring the kinetics of the reductive half-reaction at various L-proline concentrations and fitting the data to eq 1 (Figure 1). The observed values for K_d were pH-independent in the range from pH 6.46 to 9.0 (K_d = 256 ± 4 mM, 4 °C). Within this pH range, the zwitterionic form of L-proline is the predominant species in solution (pK_a = 10.6). The results clearly indicate that MSOX does not selectively bind the anionic form of L-proline since in this case the observed values for K_d should be pH-dependent. MSOX may bind the anionic form of L-proline, but the latter is not a significant species in solution within the pH range studied.

The observed values for k_{lim} were strongly pH-dependent. The rates decreased at low pH and reached a limiting value at high pH (Figure 2). The data gave a good fit to eq 2, where K_a is the ionization constant of a group in the E_{ox}•S complex that must be unprotonated for enzyme reduction to occur (pK_a = 8.02).

Absorption Spectrum of the E•S Complex Formed with MSOX and L-Proline at pH 8.0. The spectral properties of the complex formed with MSOX and L-proline at pH 8.0 provide preliminary insight regarding the nature of the ionizable group in the E_{ox}•S complex. Reduction of MSOX with L-proline is slow as compared with the corresponding oxidative half-reaction. We therefore reasoned that the oxidized enzyme•L-proline complex would be the major species formed with 600 mM L-proline in aerobic buffer at pH 8.0 (e.g., −70% E•S, with K_d = 260 mM) and should be

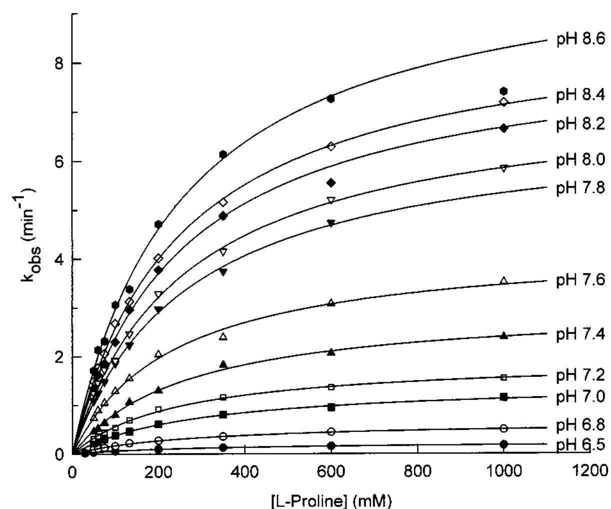


FIGURE 1: Effect of pH on the observed rate of MSOX reduction (k_{obs}) at various concentrations of L-proline. Reactions were conducted under anaerobic conditions in 100 mM potassium phosphate buffer (pH ≤ 8.0) or potassium pyrophosphate buffer (pH ≥ 8.0) at 4 °C. Reactions were initiated by rapidly tipping a small aliquot (25 μL) of a concentrated MSOX solution into a buffered solution of L-proline (575 μL) in the main compartment of the anaerobic cuvette. A single tip and return of the cuvette to the spectrophotometer was completed in less than 15 s. The solid lines are fits of the data to eq 1.

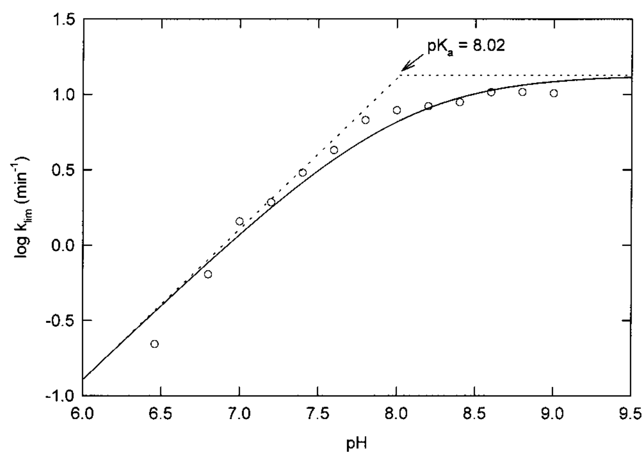


FIGURE 2: Effect of pH on the limiting rate of reduction (k_{lim}) of MSOX at saturating L-proline. Reactions were conducted and values for k_{lim} obtained as described in the legend to Figure 1. The solid line is a fit of the data (open circles) to eq 2. The dotted lines have slopes equal to +1 or 0. No further change in k_{lim} values were observed at pH > 9 (data not shown).

detectable in spectra recorded immediately after mixing (i.e., prior to consumption of dissolved oxygen). Indeed, the initial spectrum observed after L-proline addition under these conditions shows no evidence of flavin reduction but does exhibit a new band at longer wavelengths (Figure 3), a feature characteristic of a charge-transfer interaction. The position of the charge-transfer band (λ_{max} = 512 nm) was estimated from the difference spectrum (see Figure 4A). Since only the anionic form of L-proline can act as a charge transfer donor, the results indicate that the substrate must be bound, at least in part, as an anion rather than a zwitterion at pH 8.0.

At 600 mM L-proline, substrate oxidation by MSOX occurs at a rate of 5.2 min^{-1} at pH 8 and 4 °C. Aerobic turnover of 17.5 μM MSOX with 600 mM L-proline under

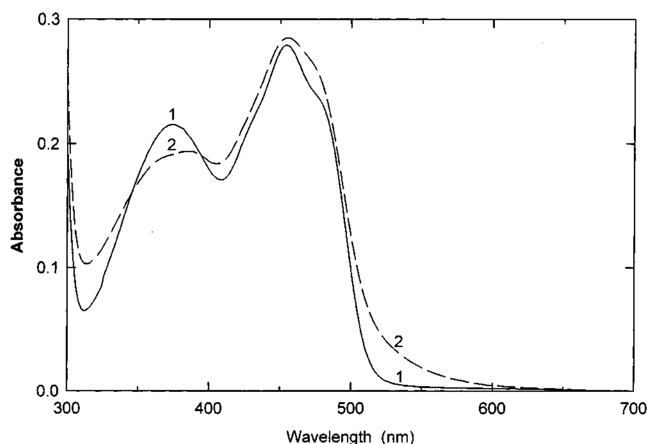


FIGURE 3: Complex formed with L-proline and MSOX at pH 8.0. Curve 1 (solid line) is uncomplexed enzyme in aerobic 50 mM potassium phosphate buffer, pH 8.0, at 4 °C. Curve 2 (dashed line) was recorded immediately after addition of 600 mM L-proline. Enzyme reduction was observed when the dissolved oxygen was consumed by turnover (data not shown).

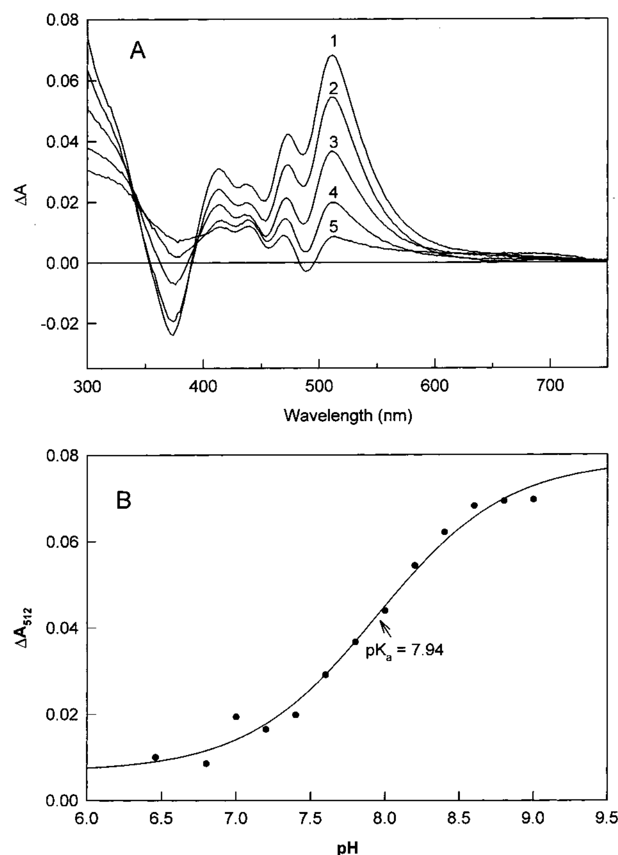
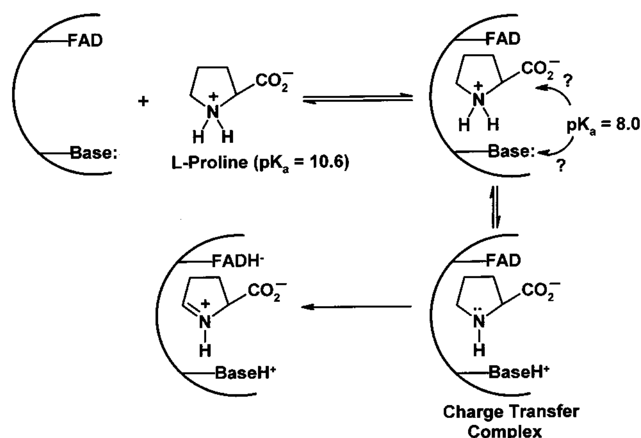


FIGURE 4: Effect of pH on charge-transfer complex formation with MSOX and L-proline. Panel A: Spectra were recorded before and immediately after mixing MSOX (17.5 μM) with 600 mM L-proline. Reactions were conducted under aerobic conditions in 100 mM potassium phosphate buffer (pH ≤ 8.0) or potassium pyrophosphate buffer (pH ≥ 8.0) at 4 °C. Curves 1−5 are difference spectra obtained for reactions at pH 8.6, 8.2, 7.8, 7.4, and 6.8, respectively. Panel B: Charge-transfer complex formation was monitored by the increase in absorption at 512 nm. The solid line is a fit of the data (closed circles) to eq 3.

these conditions will therefore generate about 73 μM product (Δ^1-pyrroline-5-carboxylate) during the time required to mix and record the long-wavelength absorption band of the E•S complex (0.8 min). The K_d for the MSOX complex with Δ^1-

Scheme 3: Electron Transfer from L-Proline to FAD in MSOX Proceeds via a Charge Transfer



pyrroline-5-carboxylate is not known but is likely be larger than that observed for pyrrole-2-carboxylate (K_d = 1.6 mM) and smaller than that observed for L-proline (K_d = 260 mM). These considerations indicate that the absorption spectrum attributed to the E•S complex is very unlikely to contain a significant contribution from a E•P complex.

Effect of pH on the Spectral Properties of the E_{ox}•S Complex Formed with MSOX and L-Proline. The absorption spectrum of MSOX was recorded before and immediately after mixing with 600 mM *L*-proline in aerobic buffers at pH 6.46−9.0, a pH range where observed values for K_d are pH-independent. Under these conditions about 70% of the enzyme is converted to the E_{ox}•S complex. Although dissociation of the E•S complex is pH-independent, development of the charge-transfer band at 512 nm was found to be pH-dependent (Figure 4A). A pK_a value of 7.94 was obtained by fitting the absorbance increase at 512 nm to a theoretical pH titration curve (Figure 4b). This value is in excellent agreement with the pK_a calculated for the E•S complex based on the limiting rate of the reductive half-reaction as a function of pH. This means that the group that must be unprotonated in the E•S complex in order for enzyme reduction to occur is likely to be the same group required to generate the reactive anionic form of the substrate that forms the charge-transfer complex.

DISCUSSION

Analysis of the anaerobic half-reaction of MSOX with L-proline as a function of pH shows that the K_d for L-proline binding is pH-independent (pH 6.46−9.0). This indicates that MSOX binds the zwitterionic form of L-proline, the predominant species in solution at neutral pH (pK_a = 10.6). Values for the limiting rate of reduction (k_{lim}) are, however, strongly pH-dependent and indicate that a group in the E_{ox}•L-proline complex (pK_a = 8.02) must be unprotonated for conversion to E_{red}•P. Charge-transfer interaction with L-proline as the donor is possible only with the anionic form of the substrate. The group with a pK_a of 8.0 is also required for conversion of L-proline from the zwitterionic to the reactive anionic form, as judged by the independently determined pK_a for charge-transfer complex formation with the MSOX flavin (pK_a = 7.94). The observation that the anionic form of L-proline with a neutral amino group is the reactive species in the reduction of MSOX is similar to that observed for other flavoenzymes that oxidize amines, including monoamine oxidase (*16*, *17*) and trimethylamine dehydrogenase (*18*, *19*). Monoamine oxidase binds the unprotonated substrate, whereas trimethylamine dehydrogenase induces a substantial shift in the substrate pK_a upon its binding to the enzyme (pK_a = 6.5 for E_{ox}•S vs pK_a = 9.3 with free S). Recent studies with D-amino acid oxidase, however, suggest that both the anionic (unprotonated amino group) and zwitterionic (protonated amino group) substrate forms are oxidized by this enzyme via a hydride transfer mechanism; slower rates are, however, reported with zwitterionic form (*20*).

It is possible that MSOX-bound L-proline exhibits a pK_a of 8.0 for conversion of the zwitterionic to the anionic form. Alternatively, conversion of the zwitterionic to the anionic form of bound L-proline may require proton transfer to a base in the E•S complex with a pK_a of 8.0 (Scheme 3). As described in the accompanying paper, evidence to discriminate between these two possibilities was sought by mutating His269, one of two potential active-site bases in MSOX.

REFERENCES

1. Kvalnes-Krick, K., and Jorns, M. S. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) pp 425−435, CRC Press, Inc., Boca Raton, FL.
2. Wagner, M. A., Khanna, P., and Jorns, M. S. (1999) *Biochemistry 38*, 5588−5595.
3. Trickey, P., Wagner, M. A., Jorns, M. S., and Mathews, F. S. (1999) *Structure 7*, 331−345.
4. Wagner, M. A., Trickey, P., Chen, Z., Mathews, F. S., and Jorns, M. S. (2000) *Biochemistry 39*, 8813−8824.
5. Chlumsky, L. J., Zhang, L., and Jorns, M. S. (1995) *J. Biol. Chem. 270*, 18252−18259.
6. Reuber, B. E., Karl, C., Reimann, S. A., Mihalik, S. J., and Dodt, G. (1997) *J. Biol. Chem. 272*, 6766−6776.
7. Silverman, R. B. (1995) *Acc. Chem. Res. 28*, 335−342.
8. Silverman, R. B., and Zelechonok, Y. (1992) *J. Org. Chem. 57*, 6373−6374.
9. Kim, J. M., Bogdan, M. A., and Mariano, P. S. (1993) *J. Am. Chem. Soc. 115*, 10591−10595.
10. Kim, J. M., Hoegy, S. E., and Mariano, P. S. (1995) *J. Am. Chem. Soc. 117*, 100−105.
11. Mattevi, A., Vanoni, M. A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., and Curti, B. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7496−7501.
12. Dinnocenzo, J. P., and Banach, T. E. (1989) *J. Am. Chem. Soc. 111*, 8646−8653.
13. Das, S., and von Sonntag, C. Z. (1986) *Z. Naturforsch. 41B*, 505−513.
14. Zhao, G., Qu, J., Davis, F. A., and Jorns, M. S. (2000) *Biochemistry 39*, 14341−14347.
15. Wagner, M. A., and Jorns, M. S. (2000) *Biochemistry 39*, 8825−8829.
16. McEwen, C. M., Sasaki, G., and Lenz, W. R. (1968) *J. Biol. Chem. 25*, 5217−5225.
17. McEwen, C. M., Sasaki, G., and Jones, D. C. (1969) *Biochemistry 8*, 3952−3962.
18. Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (2001) *J. Biol. Chem. 276*, 42887−42892.
19. Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (2001) *J. Biol. Chem. 276*, 24581−24587.
20. Harris, C. M., Pollegioni, L., and Ghisla, S. (2001) *Eur. J. Biochem. 268*, 5504−5520.

BI020285N