Bioorganic Chemistry 38 (2010) 115–119

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Characterization of active site residues of nitroalkane oxidase

Michael P. Valley [a], Nana S. Fenny [a], Shah R. Ali [a], Paul F. Fitzpatrick [b,*]

[a] Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843, USA
[b] Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 17 November 2009
Available online 28 December 2009

Keywords:
Enzyme kinetics
Flavoprotein
Isotope effects
Mutagenesis
Active site
Nitroalkane oxidase

ABSTRACT

The flavoenzyme nitroalkane oxidase catalyzes the oxidation of primary and secondary nitroalkanes to the corresponding aldehydes and ketones plus nitrite. The structure of the enzyme shows that Ser171 forms a hydrogen bond to the flavin N5, suggesting that it plays a role in catalysis. Cys397 and Tyr398 were previously identified by chemical modification as potential active site residues. To more directly probe the roles of these residues, the S171A, S171V, S171T, C397S, and Y398F enzymes have been characterized with nitroethane as substrate. The C397S and Y398 enzymes were less stable than the wild-type enzyme, and the C397S enzyme routinely contained a substoichiometric amount of FAD. Analysis of the steady-state kinetic parameters for the mutant enzymes, including deuterium isotope effects, establishes that all of the mutations result in decreases in the rate constants for removal of the substrate proton by ~5-fold and decreases in the rate constant for product release of ~2-fold. Only the S171V and S171T mutations alter the rate constant for flavin oxidation. These results establish that these residues are not involved in catalysis, but rather are required for maintaining the protein structure.

1. Introduction

The flavoenzyme nitroalkane oxidase (NAO) catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones with consumption of molecular oxygen and release of nitrite and hydrogen peroxide (Scheme 1) [1]. While there is growing evidence for hydride transfer mechanisms for the large family of flavoprotein oxidases [2–6], NAO is unusual in that it catalyzes nitroalkane oxidation through formation of a carbanion intermediate (Scheme 2) [3]. Based on analysis of the sequence of the cloned enzyme, NAO was identified as a homolog of the acyl-Co dehydrogenase (ACAD) family of flavoproteins [7]. This assignment is consistent with the similarities in the initial catalytic reactions of the two enzymes; in both cases a protein carboxylate abstracts an acidic proton from the substrate [1]. The subsequent determination of the structure of NAO [8] confirmed this assignment and provided insight into the use of this fold to catalyze the different reactions. Critically, while both the cofactors and the active site bases in NAO and ACAD occupy similar locations in the different proteins, the substrates access the active site from opposite sides of the protein [9], providing a structural basis for the reaction specificities. In addition to providing an opportunity to understand the structural basis by which a common structure can catalyze divergent reactions, NAO allows comparison of the enzyme-catalyzed reaction with a well-studied solution reaction. The comparable non-enzymatic reaction, formation of a nitroalkane anion from a nitroalkane, has long been studied as a model for proton abstraction from carbon [10,11]. Recently, study of the oxidation of nitroethane by NAO has allowed the evaluation of the contribution of quantum mechanical tunneling to the rate increase in the enzyme-catalyzed reaction [12–14].

As with many other flavoenzymes, the catalytic mechanism of NAO can be divided into oxidative and reductive half-reactions. In the reductive half-reaction, Asp402 abstracts the α-proton from the neutral nitroalkane substrate to form a nitroalkane anion which then attacks the N5 position of the FAD cofactor. The initial adduct rearranges to release nitrite and generate a reactive cationic imine species that reacts with hydroxide to eventually form reduced FAD and the aldehyde or ketone product. This cationic imine has been trapped with cyanide and its structure determined (Fig. 1), firmly establishing it as along the catalytic pathway [9,15]. In the more typical oxidative half-reaction, molecular oxygen attacks the reduced FAD to form hydrogen peroxide and regenerate the oxidized cofactor.

The knowledge of the structure of NAO has implicated several active site residues as potentially important for catalysis. The active site base of NAO, Asp402, is part of a catalytic triad that also contains Arg409 and Ser276 (Fig. 1). Mutation of any of these three residues decreases the rate constant for removal of the substrate proton by 2–3 orders of magnitude, confirming their importance

Abbreviations: NAO, nitroalkane oxidase; ACAD, acyl-CoA dehydrogenase; $K_{nitroethane}$, K_m value for nitroethane.

* Corresponding author. Address: Department of Biochemistry, UTHSCSA, MC 7760, San Antonio, TX 78229, USA.
E-mail address: fitzpatrick@biochem.uthscsa.edu (P.F. Fitzpatrick).

0045-2068/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bioorg.2009.12.004

$$RCH_2NO_2 + O_2 + H_2O \longrightarrow RCHO + NO_2^- + H_2O_2 + H^+$$

Scheme 1.

[9,16,17]. In addition, the structures of both the resting form of the enzyme and of the cyanide-trapped intermediate show that the hydroxyl of Ser171 forms a hydrogen bond with the N5 position of the flavin, suggesting it may modulate the reactivity of the flavin. A similar interaction is seen in the ACAD family, which contains a conserved threonine residue that aligns with Ser171 in NAO. Mutagenesis of this threonine to alanine in human medium-chain ACAD significantly decreases the rate constant for flavin reduction [18]. Finally, chemical modification studies have identified Cys397 [19] and Tyr398 [20] as putative active site residues in NAO. We report here the results of site-directed mutagenesis to investigate the roles these three residues play in catalysis.

2. Experimental procedures

2.1. Materials

All chemicals were purchased from Sigma–Aldrich Chemical Corp. (Milwaukee, WI). Recombinant nitroalkane oxidase was expressed and purified as previously described [7]. Mutations were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and the mutant enzymes were expressed and purified following the protocol for the wild-type enzyme. DNA sequencing of the entire coding sequence of each mutant plasmid was performed at the Laboratory for Plant Genome Technologies of Texas A&M University. Protein concentrations were determined by the method of Bradford [21] with bovine serum albumin as standard; for all kinetic measurements, enzyme concentrations were determined using an ε_{446} value of 14.2 mM^{-1} cm^{-1}, as previously described [22].

2.2. Methods

Enzyme activity was measured in 200 mM HEPES, 0.1 mM FAD, pH 8.0, 30 °C, by monitoring oxygen consumption with a computer-interfaced Hansatech Clark oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK). When varying the concentration of nitroethane, the steady-state kinetic parameters were determined at ambient oxygen concentrations. (This concentration of oxygen is 5–10 times the K_m value for oxygen for each mutant.) To vary the concentration of oxygen at a constant concentration of nitroethane, oxygen and argon were combined in different ratios with a MaxBlend low flow air/oxygen blender (Maxtec Inc., Salt Lake City, Utah), and the assay buffer was equilibrated with the gas mixture. To prevent the formation of the anionic form of the substrate, stock solutions of neutral nitroethane were prepared in dimethyl sulfoxide and assays were initiated by the addition of substrate.

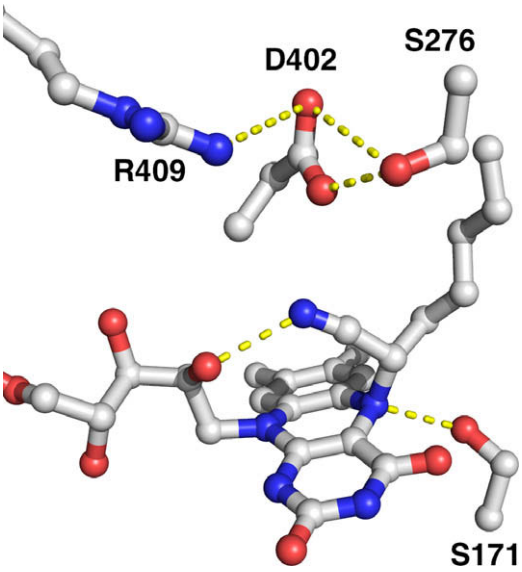
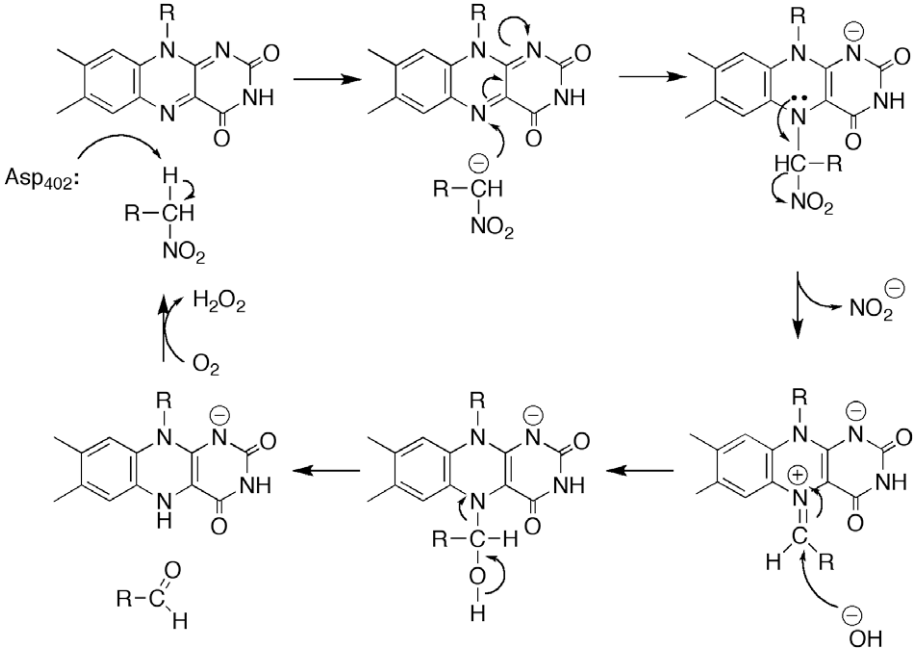


Fig. 1. Interactions in the active site of nitroalkane oxidase. The structure is that of the enzyme trapped with cyanide turning turnover with 1-nitrohexane (pdb code 3D9G) and shows the 5-cyanohexyl-FAD.



Scheme 2.

Table 1
Steady-state kinetic parameters for NAO mutant enzymes.[a]

Enzyme	k_{cat} (s^{-1})	$k_{cat}/K_{nitroethane}$ ($mM^{-1}\ s^{-1}$)	$K_{nitroethane}$ (mM)	K_{ai} (mM)	k_{cat}/K_{O_2}[c] ($mM^{-1}\ s^{-1}$)	K_{O_2} (μM)
Wild-type[b]	15 ± 1	6.3 ± 0.4	2.3 ± 0.2	25 ± 3	310 ± 30	82 ± 9
S171A	5.4 ± 0.4	2.6 ± 0.3	2.1 ± 0.4	71 ± 14	190 ± 40	29 ± 6
S171V	6.2 ± 0.1	3.2 ± 0.1	1.9 ± 0.1	238 ± 32	140 ± 7	44 ± 2
S171T	7.2 ± 0.8	3.6 ± 0.6	2.0 ± 0.5	72 ± 18	580 ± 210	11 ± 4
Y398F	10.6 ± 0.8	2.2 ± 0.18	4.8 ± 0.7	38 ± 6	390 ± 80	28 ± 6
C397S	7.3 ± 0.6	3.6 ± 0.4	2.0 ± 0.4	72 ± 16	290 ± 20	58 ± 4

[a] Conditions: pH 8.0, 30 °C.
[b] From Ref. [17], with the exception of the oxygen kinetics.
[c] Determined with 20 mM nitroethane.

2.3. Data analysis

Data were analyzed using the programs KaleidaGraph (Synergy Software) and Igor Pro (WaveMetrics, Inc., Lake Oswego, OR). Steady-state kinetic parameters were determined by fitting the data to the Michaelis–Menten equation or to Eq. (1), which includes the effect of substrate inhibition. The parameter v is the initial velocity, k_{cat} is the turnover number, K_m is the Michaelis constant, S is the substrate concentration, and K_{ai} is the substrate inhibition constant. Steady-state kinetic isotope effects were calculated from Eq. (2), where F_i is the fraction of deuterium in the substrate and $^{D}k_{cat}$ and $^{D}(k_{cat}/K_m)$ are the isotope effects on k_{cat} and k_{cat}/K_m, respectively.

$$v = k_{cat}S/(K_m + S + S^2/K_{ai}) \quad (1)$$

$$v = \frac{k_{cat}S}{K_m(1 + F_i(^{D}(k_{cat}/K_m) - 1)) + S(1 + F_i(^{D}k_{cat} - 1)) + \frac{S^2}{K_{ai}}} \quad (2)$$

3. Results

3.1. Mutagenesis of Ser171

Ser171 was mutated to alanine, valine, and threonine to probe its role in catalysis. In all cases the mutant enzymes were well-behaved and could readily be purified using the protocol developed for the wild-type enzyme. The concentration of active enzyme determined from the ε_{446} value was typically equivalent to the total protein concentration, indicating ~100% flavin occupancy. The steady-state kinetic parameters of the mutant enzymes with nitroethane as substrate are summarized in Table 1. For all three mutant enzymes, the k_{cat} values and k_{cat}/K_m values for nitroethane for the three mutant enzymes are down 2 to 3-fold from the wild-type values. The k_{cat}/K_m values for oxygen are similarly about 2-fold less for the S171A and S171V enzymes, while the value for the S171T enzyme is at least as large as the wild-type value. Deuterium kinetic isotope effects were also determined for the three mutant proteins. The $^{D}(k_{cat}/K_{nitroethane})$ values are large and comparable to that for the wild-type enzyme. The $^{D}k_{cat}$ values for the S171V and S171A enzymes show only a slight increase from the wild-type value, while this isotope effect for the S171T enzyme shows a larger increase.

3.2. Mutagenesis of Tyr398

Tyr398 was mutated to phenylalanine to probe the role of the phenol in catalysis. Flavin binding appeared to be unperturbed in the Y398F enzyme, in that the purified enzyme contained a stoichiometric amount of FAD. However, this mutant enzyme precipitated from solution over prolonged periods of time at 30 °C, and thus is less stable than the wild-type enzyme. The steady-state kinetic parameters and deuterium isotope effects with nitroethane as substrate for this mutant are given in Tables 1 and 2. The kinetic parameters for the Y398F enzyme are all close to the wild-type values, with the largest difference being a threefold decrease in the k_{cat}/K_m value for nitroethane. The isotope effects are similarly close to the wild-type values, with a small increase in the $^{D}k_{cat}$ value.

Table 2
Steady-state kinetic isotope effects for NAO mutant enzymes.[a]

Enzyme	$^{D}k_{cat}$	$^{D}(k_{cat}/K_{nitroethane})$
Wild-type[b]	1.4 ± 0.2	9.2 ± 1.1
S171A	1.9 ± 0.2	11.6 ± 2.3
S171V	1.6 ± 0.1	14.6 ± 1.3
S171T	2.4 ± 0.4	8.5 ± 2.8
Y398F	1.9 ± 0.3	10.8 ± 1.7
C397S	2.4 ± 0.3	8.5 ± 2.5

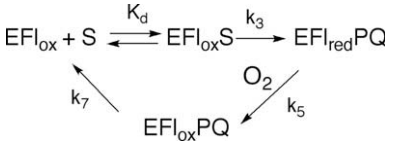
[a] Conditions: pH 8.0, 30 °C.
[b] From Ref. [17].

3.3. Mutagenesis of Cys397

Cys397 was mutated to serine to determine the role of the thiol in catalysis. Analyses of different preparations of the C397S enzyme indicated that it was 50–90% apoenzyme. This implies that the binding of flavin is perturbed by mutation of this residue. Consistent with such a structural perturbation, the C397S enzyme was less stable than the wild-type enzyme in that it more readily precipitated from solution after multiple freeze/thaw cycles. The steady-state kinetic parameters with nitroethane as substrate for C397S enzyme are summarized in Table 1. These are unaffected by the presence of apoenzyme, since all enzyme concentrations were determined from the flavin content and thus the values are for the holoenzyme only. The k_{cat} value and the k_{cat}/K_m value for nitroethane decreased about 2-fold, while the k_{cat}/K_m value for oxygen is unchanged from the wild-type values. This enzyme also shows the largest $^{D}k_{cat}$ value of any of the mutant enzymes.

4. Discussion

None of the mutations described here had large effects on any steady-state kinetic parameter. However, large changes in individual rate constants do not always have commensurate effects on steady-state kinetic parameters. This is especially the case when



Scheme 3.

Table 3
Intrinsic kinetic parameters for nitroalkane oxidase mutants.

Kinetic parameter	Wild-type NAO[a]	S171A	S171T	S171V	C397S	Y398F
K_d (mM)	14 ± 1 (48 ± 24)[b]	25 ± 9	11 ± 6	48 ± 13	11 ± 5	52 ± 21
k_3 (s^{-1})	247 ± 5 (310 ± 150)	49 ± 12	42 ± 13	74 ± 16	42 ± 10	96 ± 33
k_5 (mM^{-1} s^{-1})	310 ± 30	190 ± 40	580 ± 210	140 ± 7	290 ± 20	390 ± 80
k_7 (s^{-1})	17 (16 ± 1)	6.1 ± 0.5	8.7 ± 1.1	5.8 ± 0.4	8.8 ± 0.8	12 ± 1

[a] From Ref. [17].
[b] Values in parentheses for the wild-type enzyme were calculated from the data in Tables 1 and 2.

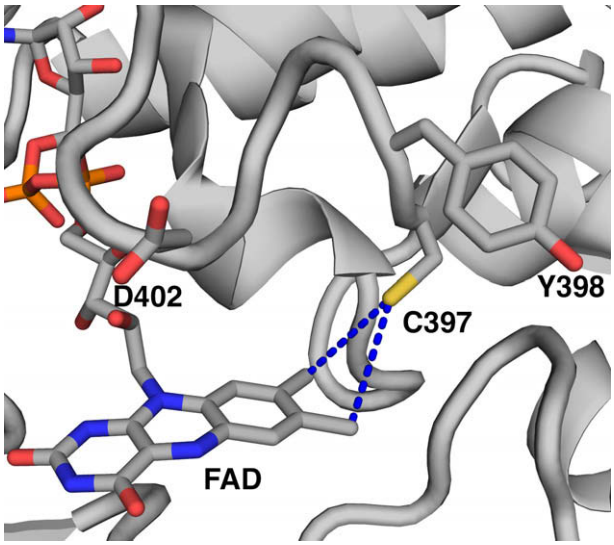


Fig. 2. Relative positions of Cys397, Tyr398, and FAD in nitroalkane oxidase.

the rate-limiting step for turnover is product release, as with NAO. A combination of steady-state kinetic parameters and kinetic isotope effects can resolve this problem, allowing one to partition the effects of the mutations among the individual steps in Scheme 3. The kinetic mechanism of NAO (Scheme 3) has been established for the wild-type enzyme using a combination of steady state and rapid reaction kinetics [17]. This mechanism provides a framework for analysis of the data for the mutant enzymes. With nitroethane as substrate, the rate-limiting step in the reductive half-reaction is cleavage of the substrate CH bond with rate constant k_3 [23]. Consequently, the deuterium isotope effect on the k_{cat}/K_m value for nitroethane, $^D(k_{cat}/K_{nitroethane})$, equals the intrinsic isotope effect for CH bond cleavage, and the k_{cat}/K_m value for nitroethane equals k_3/K_d, with K_d the dissociation constant for nitroethane. The relationship between the K_m and the K_d values is given by Eq. (3) [24]. Oxygen reacts with the reduced enzyme with second-order kinetics, with no indication of saturation at accessible levels of oxygen [17]. Such kinetics are typical of the oxygen reaction of flavoprotein oxidases [25]. As a result, the value of k_5, the rate constant for the reaction of the reduced enzyme with oxygen, is equivalent to the k_{cat}/K_m value for oxygen. The K_{O_2} value does not reflect a true binding event; rather, it equals $k_{cat}/(k_{cat}/K_{O_2})$ (Eq. (4)). For wild-type NAO, overall turnover at saturating concentration of nitroethane and oxygen is limited by product release from the oxidized enzyme, with rate constant k_7 [17]; because this step is 19-fold slower than reduction with the wild-type enzyme, the isotope effect on the k_{cat} value is small. Eq. (5) gives the relationship between the k_{cat} value and k_3 and k_7. The relationship between the isotope effect on k_{cat}, $^Dk_{cat}$, and the intrinsic isotope effect on the CH bond cleavage step, $^Dk_3$, is given by Eq. (6). These relationships allow the data of Tables 1 and 2 to be used to calculate the values of the individual rate constants in Scheme 3 for each mutant enzyme (Table 3).[1]

$$K_d = K_m^D(k_{cat}/K_m - 1)/(^Dk_{cat} - 1) \quad (3)$$

$$K_{O_2} = \frac{k_3 k_7}{(k_3 + k_7)k_5} \quad (4)$$

$$k_{cat} = k_3 k_7/(k_3 + k_7) \quad (5)$$

$$^Dk_{cat} = (^Dk_3 + k_3/k_7)/(1 + k_3/k_7) \quad (6)$$

When any of the three residues is mutated, multiple steps are affected, suggesting that the decreases in activity are due to small structural changes rather than loss of catalytically important residues. The largest effects of the mutations are on the rate constant for proton abstraction from the substrate, k_3, with smaller effects on the rate constants for product release, k_7. Given that NAO increases the rate constant for proton abstraction for the substrate by a billion-fold compared to the uncatalyzed reaction, [12], it is not surprising that even a small perturbation of the structure would decrease this rate constant. The active site of NAO is at the bottom of a long tunnel, placing the FAD N5 ~20 Å from the surface of the protein. Diffusion of the product out of the enzyme limits turnover for the wild-type enzyme; the decreases in k_7 seen here with the mutant enzymes suggests that even small disruptions in the overall protein structure can slow this movement. The effects of the mutations on the rate constant for the reaction with oxygen are smaller than those on the reductive half-reaction, with only the S171A and S171V enzymes showing any change. The lack of a significant effect of mutating Ser171 establishes that the hydrogen bond between this residue and the flavin N5 is not important for modulating the reactivity of either the reductive or oxidative half-reactions. Instead, the hydrogen bond between Ser171 and the FAD may affect the active site dynamics. This would be consistent with the introduction of larger residues, but not replacement of serine with the smaller alanine, altering the kinetics.

Cys397 and Tyr398 were identified as potential active site residues by chemical modification. The modest effects of the C397A and Y398F mutations on flavin binding and stability suggest that these mutations have rather general disrupting effects on the protein structure. The sulfur of Cys397 is 4 Å from both of the flavin methyl groups, and both Cys397 and Tyr398 are located in a helix that terminates at Asp402 (Fig. 2). Chemical modification of either and the resulting increase in bulk would be expected to significantly perturb the positioning of the active site base and the flavin, providing a reasonable explanation for the loss of activity when these residues are modified. The much smaller effects seen with the conservative mutations seen here confirm that the roles of Cys397 and Tyr398 are structural rather than catalytic.

The residues in NAO analyzed herein by site-directed mutagenesis were selected either because the structure of the protein

[1] The values in Table 3 were calculated using 9.2 ± 1.1 as the intrinsic isotope effect for all the enzymes, since the $^D(k_{cat}/K_{nitroethane})$ values for all four mutant enzymes are equivalent to the value for the wild-type enzyme. Use of the individual $^D(k_{cat}/K_{nitroethane})$ values for the mutant enzymes does not have a significant effect on the calculated values.

suggests they play a catalytic role (Ser171) or because previous solution studies suggested that they were near the active site (Cys397 and Tyr398). In all three cases, the effects of the mutations are too small to consider these residues critical for catalysis.

Acknowledgment

This research was supported in part by NIH Grant GM58698.

References

[1] P.F. Fitzpatrick, A.M. Orville, A. Nagpal, M.P. Valley, Arch. Biochem. Biophys. 433 (2005) 157–165.
[2] P.F. Fitzpatrick, Bioorg. Chem. 32 (3) (2004) 125–139.
[3] M.P. Valley, P.F. Fitzpatrick, J. Am. Chem. Soc. 23 (125) (2003) 8738–8739.
[4] P.F. Fitzpatrick, J. Labelled Compd. Radiopharmaceut. 50 (2007) 1016–1025.
[5] P.F. Fitzpatrick, Arch. Biochem. Biophys. (2010), in press.
[6] G. Gadda, Biochemistry 47 (52) (2008) 13745–13753.
[7] S.C. Daubner, G. Gadda, M.P. Valley, P.F. Fitzpatrick, Proc. Natl. Acad. Sci. USA 99 (5) (2002) 2702–2707.
[8] A. Nagpal, M.P. Valley, P.F. Fitzpatrick, A.M. Orville, Biochemistry 45 (4) (2006) 1138–1150.
[9] A. Héroux, D.M. Bozinovski, M.P. Valley, P.F. Fitzpatrick, A.M. Orville, Biochemistry 48 (15) (2009) 3407–3416.
[10] C.F. Bernasconi, Accounts Chem. Res. 25 (1992) 9–16.
[11] R.P. Bell, in: The Proton in Chemistry, second ed., Cornell University Press, Ithaca, 1973, pp. 250–296.
[12] M.P. Valley, P.F. Fitzpatrick, J. Am. Chem. Soc. 126 (20) (2004) 6244–6245.
[13] D.T. Major, D.M. York, J. Gao, J. Am. Chem. Soc. 127 (47) (2005) 16374–16375.
[14] D.T. Major, A. Heroux, A.M. Orville, M.P. Valley, P.F. Fitzpatrick, J. Gao, Proc. Natl. Acad. Sci. USA 106 (2009) 20734–20739.
[15] M.P. Valley, S.E. Tichy, P.F. Fitzpatrick, J. Am. Chem. Soc. 127 (7) (2005) 2062–2066.
[16] P.F. Fitzpatrick, M.P. Valley, D.M. Bozinovski, P.G. Shaw, A. Héroux, A.M. Orville, Biochemistry 46 (2007) 13800–13808.
[17] M.P. Valley, P.F. Fitzpatrick, Biochemistry 42 (19) (2003) 5850–8566.
[18] B. Kuchler, A.G. Abdel-Ghany, P. Bross, A. Nandy, I. Rasched, S. Ghisla, Biochem. J. 337 (Pt 2) (1999) 225–230.
[19] G. Gadda, A. Banerjee, L.J. Dangott, P.F. Fitzpatrick, J. Biol. Chem. 275 (41) (2000) 31891–31895.
[20] G. Gadda, A. Banerjee, P.F. Fitzpatrick, Biochemistry 39 (2000) 1162–1168.
[21] M.M. Bradford, Anal. Biochem. 72 (1967) 248–254.
[22] G. Gadda, P.F. Fitzpatrick, Biochemistry 37 (1998) 6154–6164.
[23] G. Gadda, D.Y. Choe, P.F. Fitzpatrick, Arch. Biochem. Biophys. 382 (1) (2000) 138–144.
[24] J.P. Klinman, R.G. Matthews, J. Am. Chem. Soc. 107 (1985) 1058–1060.
[25] V. Massey, J. Biol. Chem. 269 (1994) 22459–22462.