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Inter-subunit cross-linking of methylamine dehydrogenase by cyclopropylamine requires residue αPhe55

Dapeng Sun, Victor L. Davidson*

Department of Biochemistry, The University of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216-4505, USA

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Abstract Cyclopropylamine is a mechanism-based inhibitor of the quinoprotein methylamine dehydrogenase (MADH) from *Paracoccus denitrificans*. The resulting inactivation is accompanied by the formation of a covalent cross-link between the α and β subunits of MADH. The results of site-directed mutagenesis studies indicate that Phe55 on the α subunit is required for this process. No cross-linking is seen with αF55A or αF55I MADH mutants. In contrast, with αF55E MADH cross-linking of subunits is observed. These results suggest a novel mechanistic role for a phenylalanine residue and the possible importance of protein dynamics in this enzyme mechanism.

Key words: Quinoprotein; Tryptophan tryptophylquinone; Enzyme inhibition; Protein dynamics

1. Introduction

Methylamine dehydrogenase (MADH) from *Paracoccus denitrificans* catalyzes the oxidative deamination of methylamine to formaldehyde plus ammonia [1]. MADH possesses an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 [2]. Each β subunit of MADH possesses a tryptophan tryptophylquinone [3] (TTQ) prosthetic group (Fig. 1), which is formed by posttranslational modifications of Trp57 and Trp108 of the β subunit. The overall reaction of MADH can be separated into a reductive (Eq. 1) and oxidative (Eq. 2) half-reaction. The natural electron acceptor for MADH enzyme is a type I copper protein, amicyanin [4] (A in Eq. 2). Artificial electron acceptors such as phenazine ethosulfate (PES) can substitute for amicyanin in the oxidation of reduced MADH [5].

$$CH_3NH_3^+ + TTQ_{ox} + H_2O \rightarrow HCHO + TTQ_{red} - NH_2 + H^+ \quad (1)$$

$$TTQ_{red} - NH_2 + 2A_{ox} \rightarrow 2A_{red} + TTQ_{ox} + NH_4^+ + H^+ \quad (2)$$

It was previously shown that cyclopropylamine is a mechanism-based inhibitor of MADH [6]. The protein-bound TTQ is reduced by addition of a stoichiometric amount of cyclopropylamine, but this compound does not serve as a substrate for MADH in the steady-state kinetic assay. Time-dependent inactivation of the enzyme by cyclopropylamine was observed, but only in the presence of a reoxidant (either amicyanin or PES). This enzyme inactivation is irreversible and is believed to result from the reaction of the cyclopropanone product with the reoxidized MADH. The inactivated enzyme exhibited an altered absorption spectrum suggesting that TTQ had been modified. Electrophoretic analysis of inactivated MADH indicated that covalent cross-linking of the α and β subunits had occurred. A reaction mechanism for this inhibition was proposed [6] in which some nucleophilic residue on the α subunit attacks the cyclopropanone product of the reaction. This is followed by ring opening with nucleophilic attack of the C6 carbonyl carbon of the reoxidized TTQ. Further evidence that TTQ is modified was obtained using a redox cycling assay to test for the presence of a functional quinone after electrophoretic separation. Whereas the β subunit stained positive, the αβ cross-linked species did not [6]. Inspection of the crystal structure of MADH [2] reveals that the residue of the α subunit that is closest to the reactive carbonyl of TTQ is αPhe55. Previous studies have shown that this residue determines the substrate specificity of MADH [7,8] and also plays a role in monovalent cation binding in the active site [9]. To investigate the role of αPhe55 in the cross-linking of subunits that is a part of the mechanism-based inactivation, we have examined the reaction of cyclopropylamine with three site-directed mutants of MADH in which Phe was converted to either Ala, Ile or Glu.

2. Materials and methods

2.1. Materials

Native MADH was purified from *P. denitrificans* as described previously [5]. The site-directed mutants of MADH were expressed in *Rhodobacter sphaeroides* [10] and purified [11] as described previously. Construction of the αF55A [7], αF55I [7] and αF55E [9] mutants was as described previously.

2.2. Steady-state assay

Steady-state kinetic assays [5] were performed in 10 mM potassium phosphate, pH 7.5, at 30°C. The assay mixture contained 16 nM MADH, varied concentrations of substrates, 4.8 mM phenazine ethosulfate and 170 μM 2,6-dichlorophenolindophenol. The reaction was monitored at 600 nm to determine the rate of reduction of the latter.

2.3. Inactivation assay

Prior to being assayed, 1 μM MADH was incubated with different concentrations of cyclopropylamine in 10 mM potassium phosphate, pH 7.5, in the presence or absence of 4.8 mM PES. At different times, aliquots of the enzyme were removed from the incubation mixture and diluted 60-fold into the steady-state assay mixture.

*Corresponding author. Fax: (1)-601-984 1501.
E-mail address: vdavidson@biochem.umsmed.edu (V.L. Davidson).

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2.4. Cross-linking assay

To determine whether covalent cross-linking of MADH subunits had occurred, 10 μM MADH was incubated for 20 min at room temperature with 600 μM cyclopropylamine in the presence or absence of 4.8 mM PES. This mixture was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

3. Results and discussion

As was seen with native MADH [6], cyclopropylamine readily reduced each of the site-directed mutants of MADH. Reduction of TTQ was monitored by characteristic changes in the absorption spectrum [12] and was complete within seconds (data not shown). Despite the rapid reduction of TTQ by cyclopropylamine, no detectable activity was observed in the steady-state assay of any of the MADH mutants when cyclopropylamine was used as a substrate at concentrations up to 600 μM. This suggests that each of the MADH mutants can be inactivated by cyclopropylamine. In order to test the inhibition properties among different MADHs, both time- and cyclopropylamine concentration-dependent inhibitions were assayed. Each MADH was incubated with 4 μM cyclopropylamine (a two-fold excess to TTQ) (Fig. 2A). The αF55E MADH was inactivated at a rate substantially faster than native MADH. The αF55I MADH was inactivated, but at a rate less than native MADH. No inactivation was observed with αF55A MADH under these conditions. Time-dependent inactivation of αF55A MADH could be observed, but only at much higher concentrations of cyclopropylamine (Fig. 2B).

In order to determine whether the mechanism-based inactivation of MADH mutants was accompanied by inter-subunit cross-linking, SDS–PAGE was performed after incubation with a large excess of cyclopropylamine in the presence or absence of a reoxidant (Fig. 3). Only αF55E MADH exhibited behavior like that of native MADH. With native MADH and αF55E MADH, the cross-linking requires the presence of the reoxidant. As was noted previously [6], the maximum level of cross-linking that is observed is approximately 50% of the total protein, based on the relative intensity of the protein stain. No cross-linking between the MADH subunits can be observed in either the inactivated αF55A MADH or αF55I MADH.

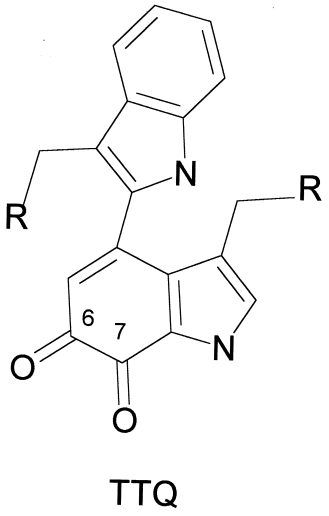

Fig. 1. The structure of oxidized TTQ. The C6 and C7 positions are labeled.

Previous studies have shown that Glu may effectively replace αPhe55 as a ligand for monovalent cation binding in MADH [9]. These results show that Glu may functionally replace αPhe55 during the mechanism-based inactivation of MADH as well. This is surprising since one would not expect the very different chemistries of Glu and Phe to lead to the same sort of cross-link. An alternative possibility is that the nature of the residue at the α55 position influences whether inactivation is followed by cross-linking between other sites. However, the α55 position seems to be the most likely site based on the crystal structure. These results highlight an unusual feature of MADH, which is to use Phe in the enzyme

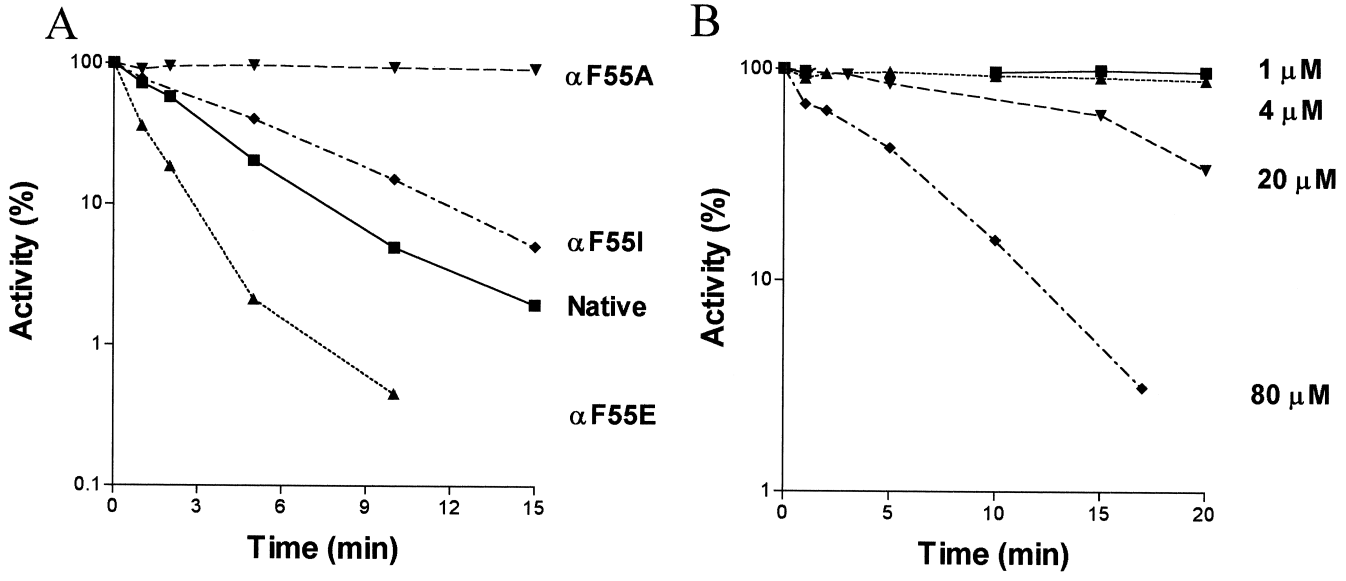

Fig. 2. A: Inactivation of native and mutant MADHs by cyclopropylamine. Each MADH was incubated in the presence of 4 μM cyclopropylamine and 4.8 mM PES. B: Concentration dependence of the inactivation of the αF55A MADH by cyclopropylamine. αF55A MADH was incubated in the presence of 4.8 mM PES and the indicated concentration of cyclopropylamine. Activity was assayed as described in Section 2.

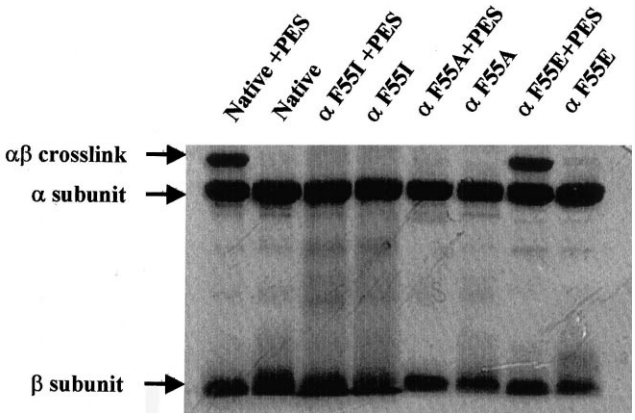

Fig. 3. Electrophoretic analysis of cyclopropylamine-inactivated MADH. Each MADH was incubated for 20 min with 600 μM cyclopropylamine in the presence or absence of 4.8 mM PES. Under these conditions each MADH is completely inactivated. Samples were subjected to SDS–PAGE on a 12% gel and stained for protein. The positions, which correspond to those of the purified α and β subunits, and the putative cross-linked αβ subunit are indicated.

active site when at least some of its functions may be performed instead by a carboxylate.

It is known that mutation of αPhe55 to Ala affects monovalent cation binding to the active site of MADH, but mutation to Glu does not [9]. To investigate whether there is a correlation between monovalent cation binding and the mechanism-based inactivation and cross-linking, additional experiments were performed. Experiments with native MADH were performed in 10 mM Bis–Tris propane buffer, which is essentially free of monovalent cations. Experiments with αF55A MADH were performed in 100 mM potassium phosphate, which should saturate the weaker monovalent cation binding site of this mutant [9]. Under these conditions results identical to those shown in Fig. 3 were obtained. Thus, effects on monovalent cation binding and mechanism-based inactivation and cross-linking appear to be two independent phenomena which involve residue α55.

One concern with the interpretation that αPhe55 and αGlu55 in αF55E MADH become covalently cross-linked with TTQ is that the distance between αPhe55 and the C6 of TTQ seems too long to be bridged by the three-carbon chain derived from cyclopropanone. This bridge is unlikely to span a distance >6 Å and the closest distance in the crystal structure is 7.4 Å [2]. One explanation for this is that during the reaction mechanism there is movement of αPhe55 and TTQ relative to each other, which brings them into closer proximity to each other. It is possible that shifts in the positions of TTQ and αPhe55 that bring these residues closer together may occur during the mechanism-based inactivation of MADH. Protein dynamics may allow transient conformations in which the reactive groups come close enough for the final step in the cross-linking to occur. These mechanisms require that the conformation of the protein active site be different after cross-linking than in its native state. In this and the previous study [6], it was noted that only about 50% of the MADH subunits get cross-linked, as judged by SDS–PAGE analysis. The enzyme, however, is completely inactive. It may be that in this $\alpha_2\beta_2$ heterotetramer, once one of the active sites becomes cross-linked, a conformational change is transmitted to the other active site, which renders it inactive and prevents mechanism-based cross-linking of the second site. This is interesting since all previous studies with MADH have indicated that the reactivities of the two active sites of the holoenzyme are identical. It is difficult to conclude from these inactivation studies that the two active sites exhibit cooperativity under normal conditions, however it does appear to exist after cross-linking occurs.

While no cross-linking was observed for αF55A MADH, substantial inactivation of αF55A MADH did occur when it was incubated with high concentrations of cyclopropylamine under these conditions. Inspection of the absorption spectrum of the inactivated αF55A MADH revealed that its spectrum had been altered in a manner similar to what is observed with the inactivated native MADH (data not shown). One explanation is that some residue on the β subunit in the active site has initiated a nucleophilic attack on the cyclopropanone product in the absence of αPhe55. Inspection of the crystal structure [2] reveals the presence of at least three potentially reactive side-chains within 6 Å of the C6 of TTQ, βAsp32, βAsp76, and βTyr119. It is possible that one of these residues could participate in the mechanism-based inactivation in the absence of αPhe55, albeit less efficiently. However, with these residues, the resulting cross-link would be intra-subunit and not evident from the analysis by SDS–PAGE.

In MADH, αPhe55 has now been shown to be capable of dictating substrate specificity [7,8] and binding monovalent cations [9]. These new results suggest another unusual role for αPhe55 as a nucleophile in this mechanism-based inactivation. These results also show that by virtue of its environment in the protein, αPhe55 is able to directly participate in a wide range of biochemical processes not typically observed for a phenylalanine residue.

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References

[1] Davidson, V.L. (2001) Adv. Protein Chem. 58, 95–140.
[2] Chen, L., Doi, M., Durley, R.C., Chistoserdov, A.Y., Lidstrom, M.E., Davidson, V.L. and Mathews, F.S. (1998) J. Mol. Biol. 276, 131–149.
[3] McIntire, W.S., Wemmer, D.E., Chistoserdov, A. and Lidstrom, M.E. (1991) Science 252, 817–824.
[4] Husain, M. and Davidson, V.L. (1985) J. Biol. Chem. 260, 14626–14629.
[5] Davidson, V.L. (1990) Methods Enzymol. 188, 241–246.
[6] Davidson, V.L. and Jones, L.H. (1991) Biochemistry 30, 1924–1928.
[7] Zhu, Z., Sun, D. and Davidson, V.L. (2000) Biochemistry 39, 11184–11186.
[8] Wang, Y., Sun, D. and Davidson, V.L. (2002) J. Biol. Chem. 277, 4119–4122.
[9] Sun, D. and Davidson, V.L. (2001) Biochemistry 40, 12285–12291.
[10] Graichen, M.E., Jones, L.H., Sharma, B.V., van Spanning, R.J., Hosler, J.P. and Davidson, V.L. (1999) J. Bacteriol. 181, 4216–4222.
[11] Zhu, Z., Jones, L.H., Graichen, M.E. and Davidson, V.L. (2000) Biochemistry 39, 8830–8836.
[12] Davidson, V.L., Brooks, H.B., Graichen, M.E., Jones, L.H. and Hyun, Y.L. (1995) Methods Enzymol. 258, 176–190.