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Identification of the lysine residue responsible for coenzyme A binding in the heterodimeric 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus tokodaii*, a thermoacidophilic archaeon, using 4-fluoro-7-nitrobenzofurazan as an affinity label

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ABSTRACT

The heterodimeric 2-oxoacid:ferredoxin oxidoreductase (StOFOR) from *Sulfolobus tokodaii*, a thermoacidophilic archaeon, was inactivated by low concentrations of 4-fluoro-7-nitrobenzofurazan (NBD-F), with concomitant increase in fluorescence in subunit-b. The inactivation was prevented by CoA, suggesting that NBD-F covalently bound to the Lys which is responsible for CoA binding. The NBD-labeled subunit-b was isolated and digested with endoproteinase Lys-C. The resulting polypeptide mixture was separated by reverse phase HPLC and the fluorescent fraction was isolated. Amino acid sequencing of the fraction revealed that it comprised a mixture of two polypeptides containing Lys125 and Lys173, respectively. Two StOFOR mutants, K125A and K173A, were constructed, expressed and purified. K125A showed a large increase in the K_m value for CoA and showed poor inactivation by NBD-F, compared with K173A and wild type StOFOR, indicating Lys125 in subunit-b is the critical residue that interacts with CoA.

1. Introduction

2-Oxoacid:ferredoxin oxidoreductase (OFOR) catalyzes the CoA-dependent oxidative decarboxylation of 2-oxoacids such as pyruvate, 2-oxoglutarate, and so on, to yield the corresponding acyl-CoAs [1,2]. The electron acceptor is mostly ferredoxin or flavodoxin. OFOR plays essential roles in central metabolism and amino acid catabolism in archaea [3,1,4–9], certain anaerobic bacteria [10–15], and a small number of amitochondrial eukaryotes [16–18]. A similar reaction found in most eukaryotes is catalyzed by the 2-oxoacid dehydrogenase multienzyme complex (ODH), which uses NAD as an electron acceptor. ODH is a large (several million Da) and complicated protein complex with three enzyme components, E1 (2-oxoacid dehydrogenase, containing thiamine pyrophosphate (TPP)), E2 (dihydrolipoamide acyltransferase, containing lipoic acid), and E3 (dihydrolipoamide dehydrogenase, containing FAD) [19,20]. In contrast, OFOR is composed of 1 to 5 subunits whose total size is 105–130 kDa, and contains 1 mol TPP and 1–3 mol iron–sulfur cluster. OFOR is a mosaic enzyme that was evolutionally constructed through shuffling, deletion, and fusion of four ancestral mini-genes [4,21,9], which correspond to a four-subunit type OFOR (subunit-α with a "YPITP motif, subunit-β with TPP- and iron–sulfur-binding motifs, subunit-γ, and subunit-δ as two-cluster intermolecular ferredoxin). OFOR is distributed to all archaea. Unlike other aerobic organisms, *Sulfolobus tokodaii*, an absolutely aerobic and thermoacidophilic archaeon, has an OFOR (StOFOR) which can use both pyruvate and 2-oxoglutarate as substrates, acetyl-CoA and succinyl-CoA being formed, respectively [9]. The residues which possibly participate in 2-oxoacid binding have been engineered and alteration of the 2-oxoacid specificity has been reported [22,23]. StOFOR is heterodimeric with a 70 kDa subunit-a and a 35 kDa subunit-b.

The crystal structures of OFOR have been reported for pyruvate: ferredoxin oxidoreductase from *Desulfovibrio africanus* (DaPFOR) as a complex with pyruvate [24] and a free radical intermediate [25]. The substrate binding site is close to TPP, and three iron–sulfur clusters are separately located about 12 Å apart. DaPFOR is a homodimer (128 kDa×2), and the monomer is divided to seven domains. Domains-III, -I, and -II correspond to subunit-a of StOFOR. Domain-VI corresponds to subunit-b of StOFOR.

So far little is known about the binding site for CoA in OFOR. Though the crystal structures of several other CoA-binding proteins have been reported, the binding modes vary [26]. The conformation of CoA in proteins varies mainly in the extent of bending of the molecule at the pyrophosphate. There are few common features in the binding modes of CoA. Due to such structural diversity we consider it is difficult to deduce the binding site for CoA in OFOR.

Abbreviations: OFOR, 2-oxoacid:ferredoxin oxidoreductase; StOFOR, 2-oxoacid: ferredoxin oxidoreductase from *Sulfolobus tokodaii*; DaPFOR, pyruvate:ferredoxin oxidoreductase from *Desulfovibrio africanus*; NBD-F, 4-fluoro-7-nitrobenzofurazan; NBD-CL, 4-chloro-7-nitrobenzofurazan; ODH, 2-oxoacid dehydrogenase multienzyme complex; CoA, coenzyme A; TPP, thiamine pyrophosphate

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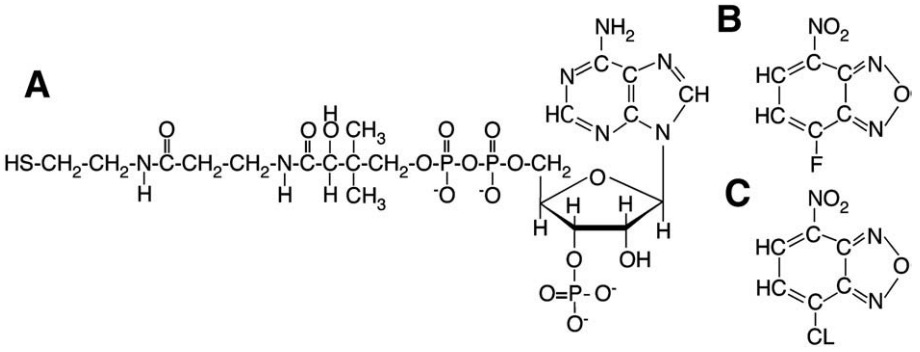

Fig. 1. Coenzyme A (A), NBD-F (B), and NBD-CL (C).

In the present study, we found that StOFOR was inactivated by low doses of 4-fluoro-7-nitrobenzofurazan (NBD-F), a reagent usually used to label the amino groups of primary and secondary amines, resulting in a highly fluorescent derivative [27,28]. 4-Chloro-7-nitrobenzofurazan (NBD-CL), with lower reactivity than NBD-F, has been used as a labeling reagent at the adenine nucleotide-binding site of various ATPases, since the molecule mimics an adenine ring (see Fig. 1) [29–33].

CoA prevented inactivation of StOFOR by NBD-F. Without CoA, NBD-labeled StOFOR showed fluorescence exclusively in subunit-b. The amino acid residue, a specific Lys, labeled by NBD-F was replaced with Ala, and the variant enzyme was characterized. Finally, our results indicate that Lys125 in the subunit-b of StOFOR is responsible for NBD- and CoA binding.

2. Materials and methods

2.1. Materials

StOFOR was purified as a recombinant enzyme encoded by a plasmid, pOFORAB, as previously reported [22,23]. 4-Fluoro-7-nitrobenzofurazan (NBD-F) (HPLC grade) was purchased from Dojindo. Endoproteinase Lys-C (sequencing grade) was purchased from Roche. All other reagents were of the highest grade available, unless otherwise mentioned.

2.2. Enzyme and protein assays

Enzyme activity was determined by pyruvate dependent reduction of methylviologen at 80 °C in a 1.5 mL cuvette containing N_2 as the gas phase, as described previously [22]. The standard assay mixture (0.5 mL) comprised 50 mM Tris–HCl, pH 8.5, 10 mM sodium pyruvate, 0.25 mM CoA, 2 mM methylviologen, and a sample enzyme. For determination of the K_m value for CoA, the CoA concentration was varied between 0.01 and 10 mM. Protein concentrations were determined by the BCA assay method (Pierce Chemical Co.) using BSA as the standard.

2.3. Inactivation of StOFOR by NBD-F at different concentrations

The enzyme (10 μM protein in 50 mM Na-borate, pH 8.0, and 0.1 mM EDTA) was incubated at 30 °C with 0, 1.0, 3.0, 7.0, 10, and 13 μM NBD-F in a final volume of 0.5 mL. After the times indicated, a 5 μL aliquot was withdrawn and mixed with 5 μL of 0.1 M Tris–HCl, pH 8.5, on ice. The mixture was subjected to the OFOR activity assay as describe above.

2.4. Prevention of NBD-F inhibition in the presence of CoA

The enzyme (10 μM protein in 50 mM Na-borate, pH 8.0, 0.1 mM EDTA, and 10 μM NBD-F) was incubated at 30 °C with 0, 1.0, 15, and 50 μM CoA in a final volume of 0.5 mL. After the times indicated, a 5 μL aliquot was withdrawn for residual OFOR activity assaying by the method described above.

2.5. Detection of the labeled subunit with NBD-F

100 μL of 20 μM NBD-F was added to 100 μL of 20 μM OFOR in 50 mM sodium-borate, pH 8.0, and then incubated at 30 °C. Samples of 10 μL each were taken at 0, 1, 5, 10, 20 and 30 min, and mixed with denaturing buffer comprising 20 mM Tris–HCl, pH 7.5, 1% SDS, 1% 2-mercaptoethanol, and 6 M urea, and then heated at 95 °C for 3 min. Denatured samples were subjected to SDS-PAGE analysis. The gel was illuminated with UV light immediately after the electrophoresis to record the fluorescence pattern, and then the gel was stained with Coomassie Brilliant Blue.

2.6. Labeling of StOFOR, separation of the NBD-labeled subunit, and proteolytic digestion

Purified StOFOR (about 2.5 mg in 100 μL) in 0.1 M sodium borate, pH 8.0, and 0.1 mM EDTA was mixed with 100 μL of 250 μM NBD-F, and then incubated for 1 min at 30 °C. Ice-cold 20 mM Tris–HCl, pH 8, was added to stop the reaction. A 10 μL aliquot of the reaction solution was taken for the activity assay, and the rest of the mixture was concentrated, and mixed with 7 M guanidium–HCl and 2 mg of dithiothreitol. The mixture was bubbled with N_2, and the protein was reduced for 1.5 h at room temperature. Subsequently, 5 mg of iodoacetic acid was added to the mixture, which was again bubbled with N_2. The protein was alkylated for 30 min in the dark at room temperature. The reaction mixture was dialyzed against 20 mM Tris–HCl overnight, and then concentrated. The sample was loaded onto a TSK G3000SW HPLC column (7.5 mm×30 cm) equilibrated with 0.1 M Na-phosphate, pH 7.0, containing 0.1% SDS to isolate the labeled subunit-b.

The subunit-b fraction was dialyzed against 25 mM Tris–HCl, pH 8.5, and 1 mM EDTA to replace the buffer salts, and then concentrated with buffer by ultrafiltration. The sample (about 350 μg in 400 μL) was mixed and reacted with endoproteinase Lys-C (ELC) (protein: ELC=100:1 by weight) for 18.5 h at 37 °C. A small aliquot was taken and subjected to size-exclusion chromatography to confirm complete digestion. The protease digest was concentrated in vacuo to dryness and then dissolved in 0.1% TFA.

2.7. Separation of the labeled polypeptide and sequence determination

The resultant solution was applied to a reversed phase column (Sensyu PEGvvASIL-120 ODS, 4.6 mm×250 mm) connected to a Waters 600E HPLC system to purify the labeled peptide. The mobile phases were A: 0.1% TFA and B: 80% CH_3CN in 0.085% TFA. The peptides were eluted with a linear 60–88% B gradient over 60 min (flow rate, 0.5 mL/min). The fragments were simultaneously detected with a Waters 2996 PDA detector and a Waters 2475 fluorescence