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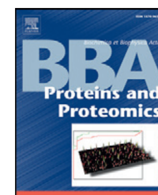

Translational misreading, amino acid misincorporation and misinterpretations. The case of the flavocytochrome b_2 H373Q variant

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ABSTRACT

Amino acid misincorporation during protein synthesis occurs naturally at a low level. Protein sequence errors, depending on the level and the nature of the misincorporation, can have various consequences. When site-directed mutagenesis is used as a tool for understanding the role of a side chain in enzyme catalysis, misincorporation in a variant with intrinsically low activity may lead to misinterpretations concerning the enzyme mechanism. We report here one more example of such a problem, dealing with flavocytochrome b_2 (Fcb2), a lactate dehydrogenase, member of a family of FMN-dependent *L*-2-hydroxy acid oxidizing enzymes. Two papers have described the properties of the Fcb2 catalytic base H373Q variant, each one using a different expression system with the same base change for the mutation. The two papers found similar apparent kinetic parameters. But the first one demonstrated the existence of a low level of histidine misincorporation, which led to an important correction of the variant residual activity (Gaume et al. (1995) Biochimie, 77, 621). The second paper did not investigate the possibility of a misincorporation (Tsai et al. (2007) Biochemistry, 46, 7844). The two papers had different mechanistic conclusions. We show here that in this case the misincorporation does not depend on the expression system. We bring the proof that Tsai et al. (2007) were led to an erroneous mechanistic conclusion for having missed the phenomenon as well as for having misinterpreted the crystal structure of the variant. This work is another illustration of the caution one should exercise when characterizing enzyme variants with low activity.

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1. Introduction

Site-directed mutagenesis of proteins constitutes nowadays an indispensable tool for analyzing and dissecting how individual amino acid side chains contribute to protein structure and stability, to enzyme mechanisms, to molecular interactions with partners, be they small molecules or macromolecules. It is known that codon translation errors, leading to misincorporation of amino acids, occur at a low rate both in nature and with recombinant systems; the error rate differs from one system to another (for references see for ex. [1,2]) and is often so low that the undesired variant is practically impossible to detect with usual methods.

Early on, Schimmel [3,4] drew attention to potential problems arising from codon misreading in the interpretation of the properties of those recombinant protein variants that have low specific activity. A low level of codon misreading, by inducing the physically undetectable presence of wild-type molecules in the purified variant enzyme preparation, may lead to erroneous conclusions as to the role of the substituted residue. An example is that of a β-lactamase, where the replacement of the active site serine with glycine yielded a somewhat active variant; changing the codon for the same mutation yielded a completely inactive enzyme [5]. Another example is that of a chloramphenicol acetyltransferase, for which the use of an affinity labeling reagent abolished the apparent residual activity of several mutations introduced at the position of the catalytic histidine [6]. In this work we provide, with the case of the flavocytochrome b_2 H373Q variant, another example of how misinterpretations can arise from unsuspected amino acid misincorporation.

Flavocytochrome b_2 (Fcb2) from *S. cerevisiae* is an *L*-lactate cytochrome *c* oxidoreductase. It has been the object of numerous studies concerning its physiological role in the mitochondrial intermembrane space, its structure and the chemical mechanism of lactate oxidation to pyruvate. The protein is composed of two domains [7,8]; the N-terminal one carries a protoheme IX and is a member of the family of b_5-like cytochromes [9]; the C-terminal one carries an FMN prosthetic group and is itself a member of a family of *L*-2-hydroxy acids oxidizing FMN-dependent enzymes, which are present in eukaryotes and prokaryotes [10]. The Fcb2 flavin oxidizes the substrate in a two-electron reaction

Abbreviations: DCIP, dichlorophenol indophenol; F1Pyr, 1-fluoropyruvate; Fcb2, flavocytochrome b_2; FDH, flavodehydrogenase domain; GOX, glycolate oxidase; LOX, lactate oxidase; LMO, lactate monooxygenase; MDH, mandelate dehydrogenase; PMSF, phenyl methane sulfonyl fluoride.

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and transfers the reducing equivalents, one at a time, to heme b_2 which delivers them to cytochrome *c*, its physiological acceptor.

Several members of the family of FMN-dependent *L*-2-hydroxy acids oxidizing enzymes have been extensively studied. Structures have been determined for WT forms of spinach and human glycolate oxidases (GOX) [10–12], of rat long chain hydroxy acid oxidase (a glycolate oxidase isozyme) [13,14], of *Aerococcus viridans* lactate oxidase (LOX) [15, 16], of *P. putida* mandelate dehydrogenase (MDH) [17] as well as for several variant forms of these enzymes. *M. smegmatis* lactate monooxygenase, (LMO, formerly called lactate oxidase), which oxidizes lactate to acetate, is the only functionally well studied family member for which no crystal structure is available [18]. Crystal structures display a high level of conservation of catalytic residues in the active site, on the FMN *si*-side at the C terminal end of the well conserved TIM barrel, with a few variations due to the different specificities.

The first mechanistic proposal for an enzyme of this family suggested that an active site base picks up the lactate C2 hydrogen as a proton, after which the carbanion would yield the electrons to the flavin [19]. Another possibility would be that the base instead picks up the hydroxyl proton, thus favoring a hydride transfer to flavin N5. The so-called carbanion mechanism is supported by numerous pieces of evidence for Fcb2 and other family members [20–25], as will be discussed later. Nevertheless, several papers concluded in recent years that the Fcb2 mechanism proceeds by hydride transfer [26,27]. The active site base in the family is a histidine (H373 in Fcb2). This histidine was mutated to glutamine in LMO [28], Fcb2 [27,29] and LOX [30], as well as to alanine, glycine and asparagine in MDH [31]. With respect to Fcb2, in a first paper [29], the apparent k_{cat} was 7600-fold lower than that of the WT enzyme. But several pieces of evidence (to be detailed below) indicated unambiguously that the enzyme preparations were contaminated by a very small amount of WT enzyme due to wobble, so that the actual activity was on the order of 10^5-fold lower than that of the WT protein. More recently, another study using a different expression system produced an enzyme with a 12,000-fold lower activity for the holoenzyme and 3600-fold lower activity for the recombinant FDH domain [27]. Although the assay conditions were somewhat different in the two studies, this decrease was comparable to that for the uncorrected data of the first report, as considered by the authors themselves [27]. Nevertheless, they did not carry out the checks described by Gaume et al. [29], and their mechanistic conclusions were different.

As just mentioned, the expression systems used in the two papers were different, even though in both cases the same codon change had been adopted: CAA for Gln versus CAT for histidine. Gaume et al. [29] used a constitutive expression system, carrying on the plasmid the complete DNA sequence for the protein precursor, even though it expresses the mature protein (for a rationale, see [32]). Tsai et al. [27] used an inducible system, with the sequence of the mature protein, as described in [33]. Could these differences lead to such discrepancies between the properties of the respective recombinant proteins? In this work, we have expressed the Fcb2 H373Q variant protein using the inducible system described by Tsai et al. [27], compared its activity under the two assay conditions, and carried out the checks described by Gaume et al. [29] for a possible contamination by the WT enzyme. The results indicate that the misincorporation is independent of the expression system. This has a consequence for the mechanistic interpretations, as will be discussed in the conclusions.

2. Materials and methods

2.1. Materials

Li^+ L-lactate was from Acros Organic, *DL*-2-hydroxy-3-butynoic acid from Tokyo Kasei Kogyo Co, monofluoropyruvic acid hydrate (F1Pyr) from Aldrich, 2,6-dichloroindophenol Na^+ salt (DCIP) from Sigma, DEAE-cellulose from Whatman, hydroxyapatite (Biogel-HTP) from Bio-Rad Laboratories. All other chemicals were of reagent grade.

2.2. Protein expression and purification

We constructed the plasmid for the inducible expression system starting from the pDSb$_2$ plasmid which had been used for constitutive expression [29,32]. The gene sequence was transferred to pET21d, the presequence of about 300 bp was removed and the His373 to Gln base change was introduced, exactly as described in [27,33]. Cultures of *E. coli* BL21 (DE3) were carried out in LB medium in the presence of 100 μg/mL ampicillin and 0.5 mg/L riboflavin at 30 °C. The cells were frozen at −75 °C until purification. The purification protocol included cell lysis in the presence of lysozyme, sonication, fractional ammonium sulfate precipitation, filtration on DEAE-cellulose followed by a gradient of phosphate buffer concentration on a hydroxyapatite column, as described in [29,34]. Flavin titration assays using the method described below (Section 2.4) indicated a flavin content of 0.76 ± 0.06 for one heme.

2.3. Enzyme assays

They were carried out with a thermostatted Uvikon XS UV/visible double beam spectrophotometer. One protocol used 0.1 M phosphate buffer, 1 mM EDTA, pH 7 and 300 μM DCIP as acceptor, at 30 °C, in 0.2 mm pathlength cuvettes [29]. The second protocol used 0.1 M phosphate buffer, 5 mM EDTA, pH 7.5 and 1 mM potassium ferricyanide as acceptor, at 25 °C [27]. In both cases the L-lactate concentration was 10 mM for the standard assay. The enzyme and reagents concentrations were determined using the following absorption coefficients: for Fcb2 $\varepsilon_{423} = 183\ mM^{-1}\ cm^{-1}$ (reduced), $\varepsilon_{413} = 129.5\ mM^{-1}\ cm^{-1}$ (oxidized), for DCIP $\Delta\varepsilon_{600} = 22\ mM^{-1}\ cm^{-1}$, for ferricyanide $\Delta\varepsilon_{420} = 1.04\ mM^{-1}\ cm^{-1}$. The kinetic parameters given in Table 1 are the result of two independent series of experiments; for each series, each substrate concentration was tested between one and three times. Experimental data were fitted to the Michaelis-Menten equation using the Kaleidagraph software for the determination of kinetic parameters, using all experimental points. Rates (k_{cat}) are expressed in terms of moles of substrate oxidized per mole flavin per second.

2.4. Flavin titrations

The flavin spectrum cannot be observed directly in Fcb2, due to its low extinction coefficient compared to the heme one. It is known that sulfite covalently adds to the oxidized flavin N5 position in a number of flavoproteins, giving a spectrum close to that of the reduced flavin [35]. The difference spectrum between the oxidized flavin and the adduct is very close to that of the flavin oxidized-reduced difference spectrum. It had been demonstrated that sulfite binds to the Fcb2 FMN without significant alteration of the heme spectum [36]. Thus, the presence of the flavin can be visualized and quantified when an Fcb2 sample is distributed between two spectrophotometer cuvettes, sulfite is added to the reference cuvette and an equal buffer volume to the sample cuvette. In this work the final sulfite concentration was 500 μM, higher than that for the WT enzyme because of a higher sulfite K_d for the H373Q variant [29]. For flavin quantification, we used the $\Delta\varepsilon_{454} = 10.5\ mM^{-1}\ cm^{-1}$ determined for the WT enzyme [36].

2.5. Inactivation reactions

The enzyme was treated with the inactivators in 0.1 M phosphate buffer pH 7 at 30 °C. Aliquots were withdrawn in the course of time and immediately diluted into the standard assay mixture for activity determinations. A second sample was incubated in parallel without reagent, as a control for spontaneous inactivation. At the end, the sample was diluted 5-fold into the phosphate buffer, and equal volumes were distributed between two spectrophotometer cuvettes for the sulfite-induced difference spectrum. For inactivation with F1Pyr, the assay mixture had DCIP as acceptor; with *DL*-2-hydroxy-3-butynoate, the

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