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Alteration of coenzyme specificity in halophilic NAD(P)$^+$ glucose dehydrogenase by site-directed mutagenesis

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ABSTRACT

Structural analysis of glucose dehydrogenase from *Haloferax mediterranei* revealed that the adenosine 2′-phosphate of NADP$^+$ was stabilized by the side chains of Arg207 and Arg208. To investigate the structural determinants for coenzyme specificity, several mutants involving residues Gly206, Arg207 and Arg208 were engineered and kinetically characterized. The single mutants G206D and R207I were less efficient with NADP$^+$ than the wild type, and the double and triple mutants G206D/R207I and G206D/R207I/R208N showed no activity with NADP$^+$.

In the single mutant G206D, the relation k_{cat}/K_{NAD^+} was 1.6 times higher than in the wild type, resulting in an enzyme that preferred NAD$^+$ over NADP$^+$. The single mutation was sufficient to modify coenzyme specificity, whereas other dehydrogenases usually required more than one or two mutations to change coenzyme specificity. However, the highest reaction rates were reached with the double mutant G206D/R207I and with coenzyme NAD$^+$, where the k_{cat} was 1.6 times higher than the k_{cat} of the wild-type enzyme with NADP$^+$. However, catalytic efficiency with NAD$^+$ was lower, as the K_m value for coenzyme was 77 times higher than the wild type with NADP$^+$.

1. Introduction

Glucose dehydrogenase (β-D-glucose: NAD(P) 1-oxidoreductase EC 1.1.1.47) catalyses the oxidation of D-glucose using NAD$^+$ or NADP$^+$ as the coenzyme. The enzyme, from diverse sources, has been utilized in NADPH regeneration in the asymmetric reduction of carbonyl compounds for the synthesis of optically active alcohols and in the enzymatic conversion of sucrose to hydrogen [1–3]. It has also been used as a biosensor on biochips for the enzymatic determination of D-glucose [4,5].

Glucose dehydrogenase from *Haloferax mediterranei* (*Hm* GDH) needs a high salt concentration (>1.5 M NaCl) for activity and stability, but is also stable in glycerol (20%, w/v) [6]. It retains its catalytic properties in reverse micelles of hexadecyltrimethylammoniumbromide (CTAB) in a cyclohexane medium, showing good stability at low salt concentrations, with the activity not dependent on salt concentration in the micelles [7]. Thus, glucose dehydrogenase from *H. mediterranei* is stable and active in different media with water restriction or in organic solvent, and is a good candidate for many different applications. Wild-type *Hm* GDH is adequate for NADPH regeneration, whereas a mutant enzyme with high activity with NAD could be very important for using this enzyme in NADH regeneration, for the enzymatic production of useful compounds, such as L-glutamate and L-carnitine [8–9].

Hm GDH is a homodimer of 39,252 Da/subunit. It can use either NAD$^+$ or NADP$^+$ as coenzyme, the K_m for NADP$^+$ being lower than that for NAD$^+$. The kinetic mechanism is sequential, with an ordered binding of NADP$^+$ and glucose followed by an ordered release of gluconolactone and NADPH [10]. The enzyme from *H. mediterranei* has been over-expressed in *E. coli* [11]. The structure of recombinant wild-type GDH from *H. mediterranei* was determined by isomorphous replacement to 2 Å, and the structure of a D38C mutant was solved to 1.6 Å. [12]. Each subunit consists of a single polypeptide chain of 357 residues that folds into two domains separated by a deep active site cleft. Of the two domains, the central domain provides the nucleotide-binding site. The topology of this domain closely resembles the classical nucleotide-binding βαβ-fold. The second domain provides the residues responsible for glucose binding and catalysis [12]. This monomer fold is similar to that of the tetrameric glucose dehydrogenase from *Thermoplasma acidophilum* [13] and to other enzymes of the medium chain dehydrogenase-reductase family [14–17]. The binding of NADP$^+$ requires stabilization of the adenosine 2′-phosphate; this is buried in a pocket formed by the side chains of conserved R207 and R208, and by a nearby cation cluster, formed by two potassium ions [12]. Residue 206 is a glycine located at the C-terminus of a β strand, which corresponds to the second β strand of the

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nucleotide-binding βαβ motif. This residue is classically considered a determinant of coenzyme specificity. In NAD^+ dependent dehydrogenases; this position is often occupied by an aspartate residue that forms double hydrogen bonds to both the 2′ and 3′ hydroxyl groups in the ribosyl moiety of NAD^+ and induces negative electrostatic potential in the binding site. In this study, we investigated, by site-directed mutagenesis, the individual and combined contributions of the adjacent residues G206, R207 and R208 to coenzyme specificity of glucose dehydrogenase, with the aim of inverting specificity and extending the potential applications of the enzyme. The first attempt to change coenzyme specificity involved making the G206D mutant. Further substitutions by uncharged residues of the residues used in the stabilization of the adenosine 2′-phosphate, Arg 207 and Arg 208, were made to analyse the importance of these residues in $NADP^+$ binding and to improve NAD^+ specificity. Three single mutants (G206D, R207I, R208N), two double mutants (G206D/R207I, G206D/R208N) and a triple mutant (G206D, R207I, R208N) were generated by site-direct mutagenesis.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutations were introduced sequentially into the glucose dehydrogenase gene cloned in the pET3a expression vector [11]. The synthetic oligonucleotide primer pairs (Bonsai Technology; Table 1) were designed to contain the desired mutation and to anneal to opposite strands of the gene. The mutagenesis procedure used followed the method of the Stratagene Quick Change kit, using *PfuUltra* DNA polymerase from Stratagene. Extension of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. Following temperature cycling, the product was treated with *Dpn* I (Fermentas). The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and was used to digest the parental DNA template and to select for mutation containing synthesized DNA. The nicked vector DNA containing the desired mutations was transformed into XL1-Blue competent cells (CNB Fermentation Service) and the isolated plasmids were sequenced to confirm mutations with a Genetic Analyzer ABI PRISM 3100 (Applied Biosystems). The mutated plasmids were transformed into expression *E. coli* cells BL21 (DE3) (CNB Fermentation Service). Expression, renaturation and purification of recombinant mutants were as previously described for wild-type halophilic GlcDH [11].

2.2. Kinetic assays and data processing

Initial velocity studies were performed in 20 mM Tris–HCl buffer pH 8.8, containing 2 M NaCl and 25 mM $MgCl_2$. The reaction was monitored by measuring the appearance of NAD(P)H at 340 nm with a Jasco V-530 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol NAD(P)H/min under the assay conditions (40 °C).

The kinetic constants were obtained from at least triplicate measurements of the initial rates at varying concentrations of D-glucose and $NAD(P)^+$. Kinetic data were fitted to the sequential ordered BiBi equation [18] with the program SigmaPlot 9.0.

$$\frac{v}{E_T} = \frac{k_{cat}AB}{K_{ia}K_b + K_aB + K_bA + AB}$$

Protein concentrations were determined by the Bradford method [19], with bovine serum albumin as a standard.

Table 1
Synthetic mutagenic primers

G206D	Forward	5′-CCTCTACTGTCTCGACCGTCGCGACCGC-3′
	Reverse	5′-GCGGTCGCGACGGTCGAGACAGTAGAGG-3′
R207I	Forward	5′-CTACTGTCTCGGCATTCGCGACCGCCCG-3′
	Reverse	5′-CGGGCGGTCGCGAATGCCGAGACAGTAG-3′
R208N	Forward	5′-GTCTCGGCCGTAACGACCGCCCGGACCC-3′
	Reverse	5′-GGGTCCGGGCGGTCGTTACGGCCGAGAC-3′
G206D/R207I	Forward	5′-CTACTGTCTCGACATTCGCGACCGCCCG-3′
	Reverse	5′-CGGGCGGTCGCGAATGTCGAGACAGTAG-3′
G206D/R207I/R208N	Forward	5′-GTCTCGACATTAACGACCGCCCGGACCC-3′
	Reverse	5′-GGGTCCGGGCGGTCGTTAATGTCGAGAC-3′

Nucleotide changes are underlined.

3. Results and discussion

3.1. Mutations for reversal coenzyme specificity

The ability of dehydrogenases to discriminate between NAD^+ and $NADP^+$ lies in the amino acid sequence of the nucleotide binding βαβ motif. This βαβ motif is centred around a highly conserved Gly–X–Gly–X–X–Gly sequence (where X is any amino acid) connecting the first β strand to the α helix. In Fig. 1, the comparison is shown of the amino acid sequence of the dinucleotide binding site of *Hm* GDH with those of other NAD^+ or $NADP^+$ specific dehydrogenases of the MDR family. The presence of an Asp residue at the C-terminal end of the second β strand is conserved in NAD^+-specific enzymes. In many $NADP^+$-specific enzymes, this residue is replaced by a smaller and neutral residue and complemented by a nearby positively charged residue that forms a positively binding pocket for the adenosine 2′-phosphate. The three-dimensional structure of the cofactor binding-site of *Hm* GDH (Fig. 2) indicates the spatial location of the residues mutated here and the interaction of Arg 207 and Arg 208 with the 2′-phosphate group of $NADP^+$ [12].

3.2. Protein properties

All the mutants were expressed as inclusion bodies and the refolding was carried out by rapid dilution in the same way as for the wild-type enzyme [11]. To assess that the enzymes reached their maximum activity in terms of proper refolding, enzyme activity was measured as a function of time after rapid dilution. The wild type and mutated enzymes behaved similarly during refolding whilst the refolding kinetics of the mutants were slower than for the wild type. Maximum activity was reached after approximately 24 h with the mutated enzymes, whereas the wild type achieved maximum activity 2 h after the rapid dilution of solubilised inclusion bodies. Once the protein was folded, the purification procedure was identical for the wild type and mutant enzymes [11]. All enzymes were purified to homogeneity, and they were stable for several weeks when stored at 4 °C.

3.3. Kinetics of wild type and mutant enzymes

The kinetic constants of the wild type and mutant forms of glucose dehydrogenase were determined with both coenzymes, NAD^+ and $NADP^+$. The mutants were assumed to have the same kinetic mechanism as the wild-type enzyme, and the kinetic data for the mutants were therefore adjusted to the BiBi sequential equation. The kinetic constants for the enzymes are compared in Table 2.

The K_m value of the wild-type enzyme was 11-fold lower for $NADP^+$ than for NAD^+, indicating that the enzyme has a strong preference for $NADP^+$. The single substitution G206D increased the K_m 74-fold for $NADP^+$ and decreased k_{cat} 2-fold, resulting in a 150-fold decrease in the k_{cat}/K_m when using $NADP^+$. This was to be expected

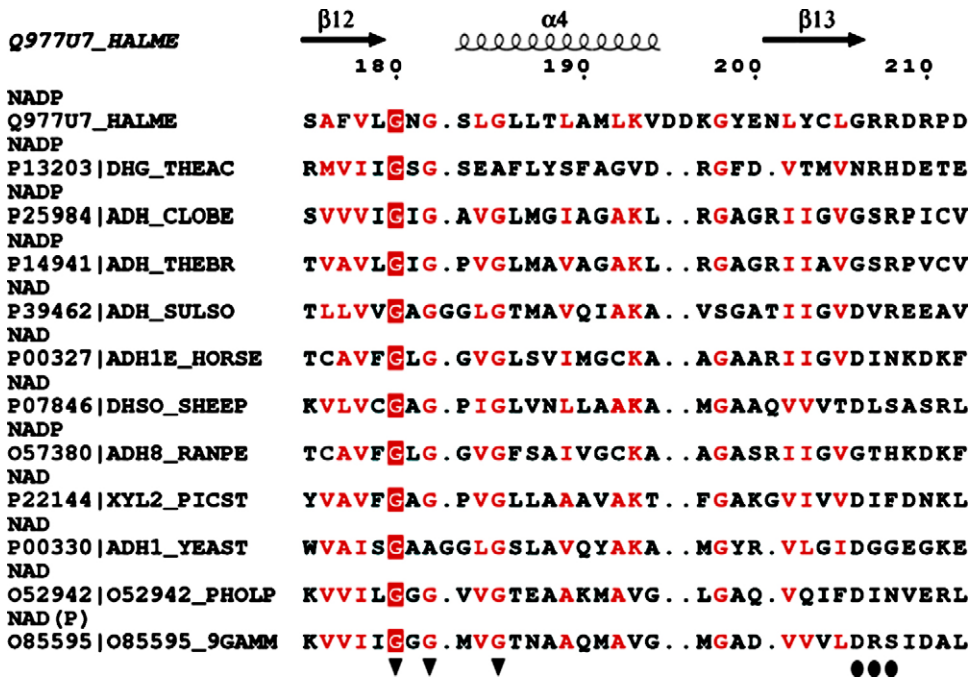

Fig. 1. Aligned amino acid sequences of the nucleotide binding βαβ motif of NAD(P) dependent dehydrogenases. In the first line the secondary structure of *H. mediterranei* glucose dehydrogenase is shown. The conserved glycine residues in nucleotide binding motif are marked (▼). Mutated residues in *Hm* glucose dehydrogenase and the equivalent position in the other enzymes are marked (●). The residues that are identical in all sequences in the alignment are in white characters and boxed in red. The conservative replacements are in red characters. The proteins are designed by the entry name and accession number of UniProt database. Q977U7: *H. mediterranei* glucose dehydrogenase, P13203: *T. acidophilum* glucose dehydrogenase, P25984: *Clostridium beijerinckii* alcohol dehydrogenase, P14941: *Thermoanaerobium brockii* alcohol dehydrogenase, P39462: *Sulfolobus solfataricus* alcohol dehydrogenase, P00327: *Equus caballus* (Horse) alcohol dehydrogenase E chain, P07846: *Ovis aries* (Sheep) sorbitol dehydrogenase, O57380: *Rana perezi* (Perez's frog) (Western Mediterranean green frog) alcohol dehydrogenase, P22144: *Pichia stipitis* (yeast) xylitol dehydrogenase, P00330: *Saccharomyces cerevisiae* (Baker's yeast) alcohol dehydrogenase 1, O52942: *Phormidium lapideum* alanine dehydrogenase, O85595: *Shewanella* sp. Ac10 alanine dehydrogenase, P31655: *Eucalyptus gunnii* (Cider tree) cinnamyl alcohol dehydrogenase 2.

as the negative charge of Asp 206 would be likely to repel the adenosine 2′-phosphate of NADP$^+$. This single substitution had a positive effect on catalysis with NAD$^+$. In NAD$^+$ dependent enzymes, an Asp residue in this position confers specificity towards NAD$^+$ by the bidentate hydrogen bonding with the 2′ and 3′ hydroxyl groups of the adenosine of NAD$^+$. The K_m in the presence of NAD$^+$ was similar to that of the wild type, but k_{cat} reported a 2-fold increase. The G206D mutant preferred NAD$^+$ to NADP$^+$, showing a k_{cat} value with NAD$^+$ similar to that of the wild-type enzyme with NADP$^+$; however, the k_{cat}/K_m ratio was still better in the wild-type enzyme with NADP$^+$.

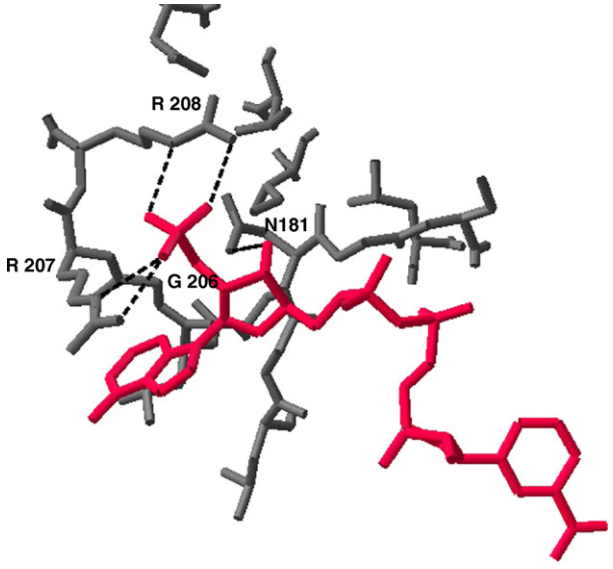

Fig. 2. View of NADP$^+$ bound to wild-type glucose dehydrogenase. Interaction through hydrogen bonds is represented with dotted lines. Crystal structure of glucose dehydrogenase from *Haloferax mediterranei* (Protein Data Bank code 2B5V, Ref. [10]).

The single mutant R207I showed an increase of 48 times in K_m value with NADP$^+$ when compared with the wild type; this again was as expected considering the role of Arg 207 in the stabilization of the negative charge of the adenosine 2′-phosphate group of NADP$^+$. This increase was accompanied by a decrease in k_{cat}, which clearly makes the R207I mutant less efficient in catalysis with NADP$^+$. For NAD$^+$ the K_m value also increased but at a ratio of 3 times, much lower than the K_m increase with NADP$^+$. The R207I substitution also makes the enzyme less efficient with NAD$^+$, with a decrease of 4 times in k_{cat}/K_m; this substitution also increases the K_m for glucose. A similar effect was even more pronounced in the single substitution R208N, in which saturation with glucose cannot be achieved, and attempts to calculate K_m and k_{cat} with both coenzymes led to very high standard deviation values.

The activity of the G206D/R207I double mutant with NADP$^+$ was very low (almost undetectable), and as such the kinetic parameters could not be calculated; however, when the coenzyme NAD$^+$ was incubated with this double mutant, it reached the highest k_{cat} value, between 1.5 and 2 times higher than the k_{cat} of the wild-type enzyme with NADP$^+$, and between 3 and 4 times higher than the k_{cat} of the wild type with NAD$^+$. These values indicate that the local rearrangement of the active centre due to the mutations makes catalysis more efficient. The dissociation constant for NAD$^+$ in the double mutant decreased 1.7-fold in comparison with $K_{iNAD}{}^+$ in the

Table 2
Kinetic constants of wild type and mutant glucose dehydrogenases

	$NADP^+$			NAD^+				
	Wild-type	G206D	R207I	Wild type	G206D	R207I	G206D/R207I	G206D/R207I/R208N
$K_{iNAD(P)^+}$ (mM)	0.09 ± 0.02	0.69 ± 0.10	1.3 ± 0.5	2.0 ± 0.2	1.0 ± 0.2	2.1 ± 0.9	1.17 ± 0.13	1.7 ± 0.4
$K_{mNAD(P)^+}$ (mM)	0.035 ± 0.009	2.6 ± 0.4	1.7 ± 0.3	0.40 ± 0.12	0.49 ± 0.16	1.3 ± 0.4	2.7 ± 0.6	1.3 ± 0.3
$K_{mglucose}$ (mM)	2.8 ± 0.3	52 ± 8	24 ± 8	12.9 ± 1.4	15 ± 4	32 ± 13	80 ± 14	57 ± 11
k_{cat}[a] (min^{-1}) ($\times 10^{-3}$)	31.1 ± 1.2	15.7 ± 1.5	11.8 ± 1.5	15.8 ± 0.9	34 ± 5	4.3 ± 0.8	54 ± 7	2.8 ± 0.3
$k_{cat}/K_{mNAD(P)^+}$ (mM^{-1} min^{-1}) ($\times 10^{-3}$)	900 ± 30	6.0 ± 1.5	7 ± 2	44 ± 17	70 ± 30	3.3 ± 0,6	20 ± 7	2.1 ± 0.7
$k_{cat}/K_{mglucose}$ (mM^{-1} min^{-1}) ($\times 10^{-3}$)	11.1 ± 1.6	0.30 ± 0.07	0.5 ± 0.2	1.2 ± 0.2	2.3 ± 0.9	0.13 ± 0.08	0.7 ± 0.2	0.049 ± 0.015

Substrate concentrations range used to determine kinetic parameters: Wild-type: [glucose] = 2–20 mM, [$NADP^+$] = 0.04–0.2 mM, [NAD^+] = 0.286–1 mM; G206D: [NAD^+] = 0.286–1 mM, [glucose] = 2.5–20 mM; [$NADP^+$] = 0.286–4 mM, [glucose] = 2.5–40 mM; R207I: [NAD^+] = 0.8–2 mM, [glucose] = 25–100 mM; [$NADP^+$] = 0.4–2 mM, [glucose] = 20–100 mM; G206D/R207I: [NAD^+] = 0.4–4 mM, [glucose] = 10–100 mM; G206D/R207I/R208N: [NAD^+] = 0.8–4 mM, [glucose] = 16.67–100 mM.

[a] k_{cat} values refer to the glucose dehydrogenase dimmer kinetic parameters are expressed ± standard deviation.

wild type, but the K_{mNAD^+} value of NAD^+ registered a 2-fold increase. The G206D/R207I/R208N triple substitution produced an inactive enzyme with $NADP^+$, confirming that these two arginines are necessary for $NADP^+$ stabilization. Regarding the kinetic parameters with NAD^+, as in the double mutant G206D/R207I, the K_m of both substrates was higher than in the wild type, but in the triple mutant the k_{cat} was also lower, and was the worst catalyst.

In contrast with our results, in alcohol dehydrogenase from gastric tissues of *Rana perezi* (UniProt accession number O57380), the complete reversal of coenzyme specificity from NADP(H) to NAD(H) was reached with the concerted mutation of three residues G223D/T224I/H225N [20]. The alignment with the equivalent sequence in glucose dehydrogenase is shown in Fig. 1. The single mutation G223D had no effect on catalysis with NAD^+ and the double mutant G223D/T224I was a better catalyst than the single mutant with NAD^+, but worst than the triple mutant. The great increase in the k_{cat} observed with the glucose dehydrogenase double mutant was not observed in alcohol dehydrogenase. It appears that one or two substitutions in alcohol dehydrogenase were not sufficient to transform coenzyme specificity, whereas multiple substitutions could be effective [20]. The same but reverse effect was observed with NAD^+-dependent xylitol dehydrogenase (UniProt P22144), in that the reverse of coenzyme specificity from NAD^+ to $NADP^+$ was achieved with the triple mutant D207A/I208R/F209S [21]. In yeast alcohol dehydrogenase, a NAD^+ specific enzyme (UniProt P00330), the D201G substitution produces an enzyme with low activity with $NADP^+$, but the G203R substitution neither affects affinity for NAD^+ or NADH nor enables reactivity with $NADP^+$, and the D201G/G203R enzyme has kinetic characteristics similar to the single D201G enzyme. Arg 203 should be able to interact with the 2′-phosphate, but it seems that a greater change is required in the amino acid sequence to transform the specificity [22].

Although there are some examples of a coenzyme specificity change from NAD^+ to $NADP^+$ with only one mutation, it seems that the specificity change from $NADP^+$ to NAD^+ is more difficult to reach with single substitutions. Alanine dehydrogenase from the cyanobacterium *Phormidium lapideum* (UniProt O52942) is strictly NAD^+ specific. This enzyme has the sequence 197-DIN-199. The same enzyme from the psychrophile *Shewanella* sp. (UniProt O85595) exhibits dual specificity for NAD^+ and $NADP^+$, and the equivalent sequence is 198-DRS-200 (the sequence comparison is shown in Fig. 1). A single mutant I198R of *P. lapideum* alanine dehydrogenase enabled this enzyme to use $NADP^+$ as a cofactor, and a single mutant R199I of the *Shewanella* alanine dehydrogenase showed strict coenzyme specificity toward NAD^+, but the k_{cat} was approximately 10 times lower than that of the wild type. The *Shewanella* alanine dehydrogenase was converted to a series of enzymes with improved preference for $NADP^+$ by replacing the D198 by G, A, V or L [23]. However, in cinnamyl alcohol dehydrogenase, an $NADP^+$-dependent enzyme (UniProt P31655), the mutation S212D did not change coenzyme specificity [24]. The equivalent substitution in *Haloferax* glucose dehydrogenase (G206D) allows the enzyme to use NAD^+ with higher specificity than in the wild type. This study shows that G206, R207 and R208 are determinant for coenzyme specificity in *Hm* glucose dehydrogenase. The substitution G206D hampered the binding of $NADP^+$ and increased by a factor of two the activity with NAD^+, resulting in an enzyme that preferred NAD^+ over $NADP^+$. Double mutation G206D/R207I was enough to make an unproductive enzyme with $NADP^+$, although the more important findings were that in double mutant G206D/R207I, the specific activity of the enzyme with NAD^+ was almost twice than in the wild type with $NADP^+$, though the k_{cat}/K_m ratio was low due to the increase of K_m. In this sense, as some authors point out [25], we have to be cautious to interpret the k_{cat}/K_m ratio as catalytic efficiency, since at certain substrate concentrations the wild-type glucose dehydrogenase catalyzes the oxidation of glucose, using $NADP^+$ as a coenzyme at a lower rate than double mutant in the presence of NAD^+

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