Purification of 6-phosphogluconate dehydrogenase

Lab report
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Abstract

The purpose of this work is to determine an estimate of the contribution of the proposed residues of 6-phosphogluconate dehydrogenase involved in binding of the phosphate-terminus of 6-phosphogluconate. Therefore these residues will be mutated to Ala and the kinetic properties of these mutants are compared to the wild-type enzyme. Therefore a small amount of fairly clean enzyme (~90% pure) should be sufficient. Unfortunelly the enzyme is not very stable, so that all purification steps are performed in the cold room and the pure protein is stored as precipitate in a high-salt buffer.

This paper describes the purification of the T262A mutant as well as the control purification of the wt *enzyme.*

Introduction: 6PGDH

6-Phosphogluconate dehydrogenase (6PGDH; EC1.1.1.44) is involved in the pentose phosphate pathway. It catalyzes the oxidative decarboxylation of 6-phosphogluconate (6PG) to ribulose-5-phosphate (Ru5P) and CO_2 while reducing $NADP^+$ to NADPH. Ribulose-5-phosphate is isomerized to ribose-5-phosphate (R5P), a precursor in the synthesis of nucleotides, by ribulose-5-phosphate isomerase in the next step of the pentose phosphate pathway.

What is special about 6PGDH compared to other oxidative carboxylates is that it has no requirement for a divalent metal ion to polarize the carbonyl and withdraw electrons from 6PG's β-γ bond [1,2]. Following this discovery a general acid – general base chemical mechanism was proposed [Fig. 1].

This mechanism could be proved using extensive kinetic and chemical studies, especially steady state kinetics, initial velocity studies and isotope effects.

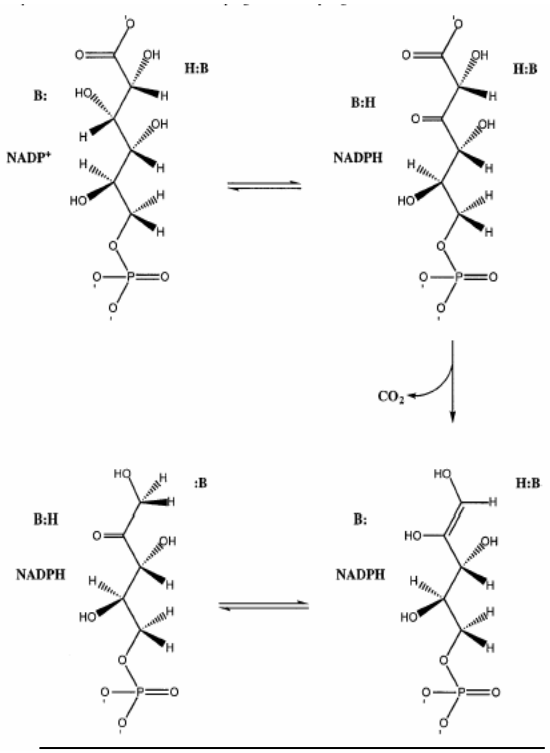

Figure 1: chemical mechanism

Due to the known crystal structure of the apoenzyme as well as forms with the substrate, co factor and inhibitors bound to it for both the sheep liver and the *Candida utilis* 6PGDH, the most likely candidates for the general acid and general base could be determinate as Glu190 and Lys183, respectively.

Using these crystal structures, the residues most likely binding the substrate at its phosphate terminus were also proposed. Each of the four residues Tyr191, Lys260, Thr262 and Arg446 appear to be in range to interact with the phosphate. Here R446 is an exception because it belongs to the second subunit of the homodimeric native form of 6PGDH. This is possible because the C-terminal "tail" of one subunit reaches through the other one to participate in the other subunits active site.

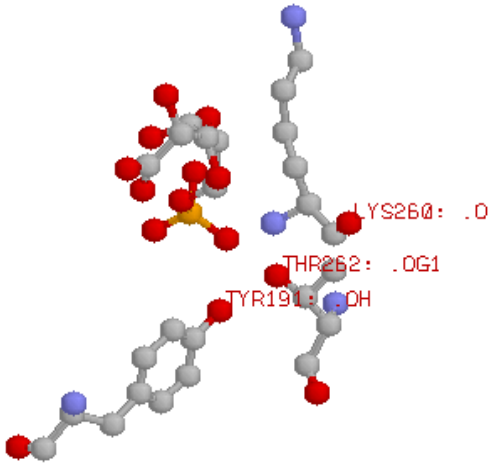

Figure 2: binding at P-terminus

My task is now to determine if these assumptions are correct and if so which estimated influence each one of them has on the specific binding of the substrate.

Therefore I make mutants in which I change every of these residues to Ala, on by the time, and perform the same kinetic experiments as where used to examine the kinetic mechanism of the *wt* enzyme.

Cloning of the *wt* sheep liver 6PGDH cDNA into the vector pAlter-1 was performed as described in reference [5]. The resulting strain, pPGDH.LC5, whose advantage is the 6xHis tag at the N-terminus of the 6PGDH sequence, was used as template for my mutations. The addition of a N-terminal 6xHis tag to the enzyme is possible because the N-terminus is not involved in either the protein folding nor in the function of the enzyme.

Tthe QuikChange™ kit was used o perform site-directed mutagenesis. I designed oligonucleotide primers containing the desired mutation and amplified the cDNA purified from the pPGDH.LC5 strain by PCR. The resulting DNA sequence was inserted into the plasmid pQE30. pQE30 is a low-copy plasmid, based on the T5 promoter. It adds a n-terminal 6xHis-tag to the desired protein, offering the possibility to use a Ni-NTA column for purification. The resulting plasmid was expressed in the M15[pREP4] strain of *E.coli*. This strain contains another plasmid, pREP4, encoding the *lac* repressor protein. This protein represses the transcription of the T5 promoter, enabling control of expression. This is useful in the case the inserted sequence is toxic to the cell. To start expression isopropyl-β-D-thiogalactoside (IPTG), which inactivates the *lac* repressor protein, is added [6].

The mutation used for the expression described in this paper is changing the T262 to A (T262A)

Methods: purification [3]

Using QIAexpress kit

Solutions:

• sonication buffer:	50 mM	sodium phosphate (pH 8.0)	
	300 mM	NaCl	
	10 mM	*β*-mercaptoethanol	
• SDS-running buffer:	25mM	TRIS	
	250mM	glycine	
	0.5%	SDS	
• SDS staining solution:	0.4% (2.5mg/ml)	Coomassie® Brilliant Blue G-250	
	1.74 M (10%)	acetic acid in 50% methanol	
• SDS destaining solution:	1.74 M (10%)	acetic acid in 50% methanol	

Expression:

Using 100µl freshly grown M15 competent cells 50µl of 1:50 diluted mutant DNA were added and incubated on ice for 30min, then heat-shocked at 42°C for 2min and then plated on LB-Agar containing 100µg/ml ampicilin and 25 µg/ml kanamycin. It is necessary to use both antibiotics, because the pQE30 plasmid containing our protein sequence is ampicilin resistant, the pREP4 plasmid encoding the *lac* repressor protein is resistant against kanamycin. The plate was incubated at 37°C over night.

The next day one single colony was picked and transferred to 10ml of LB-broth, containing 100µg/ml ampicilin and 25 µg/ml kanamycin. The culture was allowed to grow over night.

This culture was added to 1l of fresh LB-broth, containing the same amount of antibiotics, and allowed to grow at 30°C until the OD_{600} reached ~0.5. Then the culture was induced using IPTG to a final concentration of 0.5mM and allowed to grow for additional 5h. After this time the cells were harvested by centrifugation at 4000rpm

(Beckmann J6-HS centrifuge; Beckmann JS-4.2 rotor) for 15min. The pellet was stored at -80°C.

Purification:

The cell pellet was thawed on ice and diluted in 3 volumes of sonication buffer. Using a small tipped sonication probe the suspension was sonicated with a Misonix SonicatorXL™ five times for 15sec at the highest level, each burst followed by a 45sec cooling period. The solution was centrifuged 10.000rpm (Beckmann J2-HS centrifuge; Beckmann SA-20 rotor) for 20min to pellet the cell debris. In the meantime the Ni-NTA resin, originally stored in 30% ethanol, was equilibrated in sonication buffer by gentle centrifugation at ~500rpm (Beckmann J6-HS centrifuge; Beckmann JS-4.2 rotor), discarding the supernatant and replacing it with fresh buffer (1x column volume, 3ml), gentle mixing and centrifugation as before. This procedure was repeated 5times.

The supernatant from the sonication was added directly to the equilibrated resin and gently shaked for 10min at 4°C. Then the resin was poured into a small column and washed with 6 column volumes of sonication buffer to get rid of cell components without 6xHis tag. Afterwards the bound proteins were eluted with a 4-step gradient of imidazol (pH8) in sonication buffer from 0.1M to 0.4M, increasing 0.1M a time. Therefore three column volumes were used end every 1ml collected in a separate tube. The protein containing tubes were detected using the Bradford assay [4] (200µl Bradford reagent, 780µl ddH_2O, 20µl fraction) and measuring the OD_{595} in a Beckmann DU 640 photometer. The fraction containing 6PGDH was found at the first elution of 0.2mM imidazol.

The found fraction was precipitated by adding ammonium phosphate to a final concentration of 75% saturation (476mg ammonium sulfate per milliliter solution) in order to keep the enzyme stable, and stored at 4°C.

To analyze purity via SDS-PAGE we poured a 0.1% SDS containing 12% PAGE gel and loaded our first 18 fractions, accompanied by a solution of our pellet 1:3 in 3x loading buffer as well as a molecular weight marker. The gel was run at a constant current of 30mA for about 1.5h. Then it was stained for two hours in SDS staining solution, washed in dH_2O till the water was not stained anymore and afterwards destained over night in SDS destaining solution.

An activity assay was also performed. Therefore several concentrations of the enzyme where added to 1ml of 100mM HEPES buffer (pH7), containing NADP and 6-phosphogluconic acid at saturating concentrations of 1mM and 2mM, respectively. Then the generation of NADPH at 340nm was measured each 3sec for 5min in a Beckmann DU 640 photometer at a constant temperature of 25°C in a 1cm/1ml quartz cuvette. The slope of the resulting graph gives the rate of the reaction.

Results: purification T262A

After some problems with growing the culture to express the protein we finally got it to yield an acceptable amount of cells. The first real indicium that we got our protein was given by the Bradford assay. The values for the OD_{595} (Table 1) show that we first wash of the cell content, then elute all proteins loosely bound to the Ni-NTA and finally at an imidazol concentration of 0.2M we eluted our protein. The imidazol concentration equals this found previously by other researches working on 6PGDH.

The fractions were run on an SDS-PAGE gel to determine weather we got our favorite protein and if so how pure it is (Figure 3). Unfortunelly we couldn't see our molecular weight markers. Other Lab members told us that the commercial pre-stained marker we used is way to weak so that it usually is not seen on the gel. But the gel showed a good purity for our favored fraction and we are running a second SDS-PAGE with another marker and two different concentrations of this fraction to determine it is the enzyme we are looking for.

Fraction	*OD_{595}*	*Conc. µg/ml*
Wash 1	1.030	~1000
Wash 2	0.753	750
Wash 3	0.168	166
Wash 6	0.008	-
0.1M 1	0.011	-
0.1M 2	0.426	420
0.1M 3	1.945	-
0.1M 4	0.623	620
0.1M 5	0.179	177
0.1M 6	0.114	113
0.2M 1	0.110	109
0.2M 2	0.218	216
0.2M 3	0.619	617
0.2M 4	0.133	130

Table 1: Bradford assay T262A

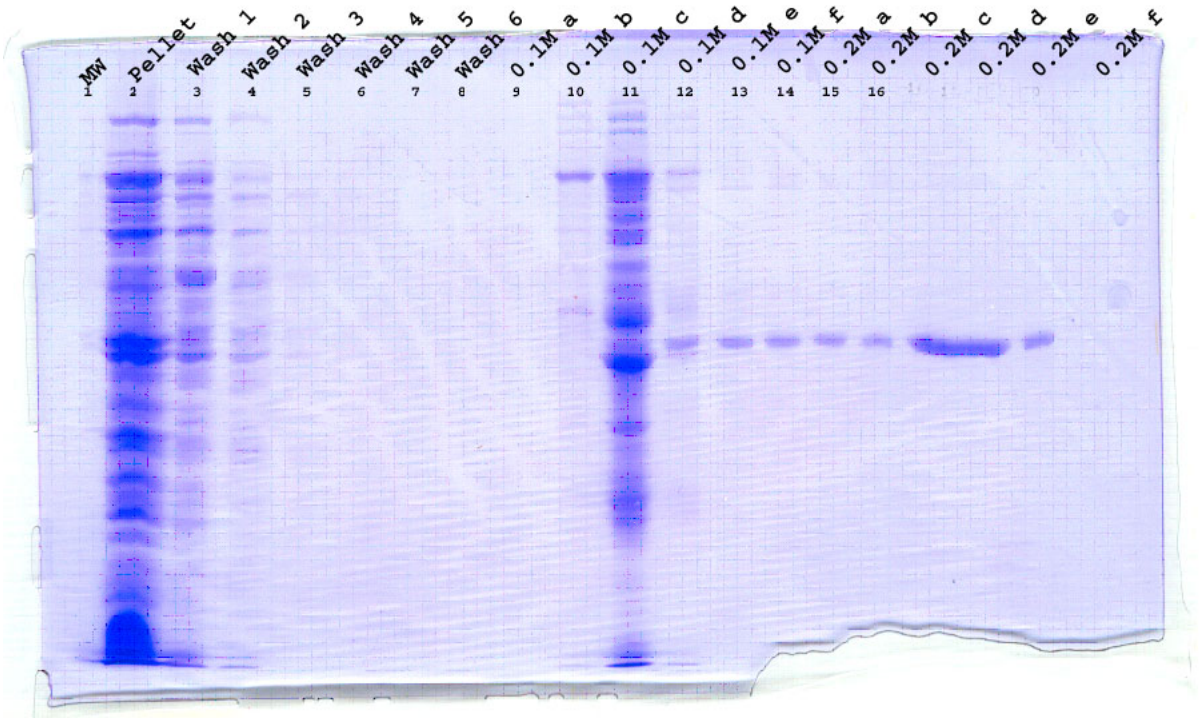

Figure 3: SDS-PAGE of T262A enzyme fractions

This time we were able to judge the molecular weight of our protein. The band was at the 51kDa subunit weight of 6PGDH. Because there were no additional bands on the SDS-Page for

the chosen fraction, we can assume that the protein is pure more than 90%, what is absolute sufficient for the planed experiments. Unfortunelly the purified protein did not show any activity, even in high concentrations (0.9µg/ml; 1.8 µg/ml; 3.6 µg/ml; 7.2 µg/ml; 18 µg/ml). Because it is a mutant affecting the active site of the enzyme, this does not necessarily mean that the expression/purification did not work. To ensure there is no error in the procedure, we purified the wild type 6PGDH directly from the pPGDH.LC5 strain using the same method as for the T262A mutant.

Results: purification wt 6PGDH

To determine the lack of activity of the enzyme isn't a problem of the method we purified the wt enzyme. Therefore we grew up a tip of the glycerol stock of the p6PGDH.LC5 strain over night in 20ml LB-medium (ampicilin/kanamycin) as before. Then we diluted this culture 1:100 in fresh LB-medium (amp/kan) as before and then followed the same protocol as for the mutant enzyme. In order to reuse the Ni-NTA resin it was washed with sonication buffer and ddH_2O and then regenerated in 0.5M NaOH for 30min. Afterwards it was washed in ddH_2O and equilibrated in sonication buffer as before.

For all fractions coming off the column the protein concentration was determined using the Bradford assay (Table 2, lane 2). For all important fractions the activity was measured right after the collecting of the fraction was finished (Table 2, lane 3, a x

Fraction	*Conc. [µg/ml]*	*Activity [U/min]*
Lysate	2254.98	25.966
Supernatant	2269.39	24.383
Flowthro.	1568.87	x
Wash 1	1828.60	x
Wash 2	443.23	x
Wash 3	67.64	x
Wash 4	23.67	x
0.1M 1	861.60	2.615
0.1M 2	2588.59	-
0.1M 3	2092.33	-
0.1M 4	729.97	-
0.1M 5	189.95	-
0.2M 1	90.97	-
0.2M 2	155.51	-
0.2M 3	339.62	35.447
0.2M 4	271.94	29.925
0.2M 5	184.24	15.779
0.2M 6	60.80	-
0.2M 7	0	0

Table 2: *wt* 6PGDH

means no assay performed, - stands for short assay w/ activity detected). Judging from the activity assay we have a small amount of our enzyme in every fraction till the last 0.2M imidazol elution. Afterwards no protein came of the column anymore.

An SDS-PAGE (as before) was run for most of the fractions (Figures 4&5), showing that indeed the 6PGDH was eluted in small amounts through out the elution process, but the biggest amount still eluted at the 0.2M imidazol elution step in a very high purity. This time we pooled the three best fractions (0.2M III, IV, and V) and precipitated the enzyme with 75% saturation of ammonium sulfate.

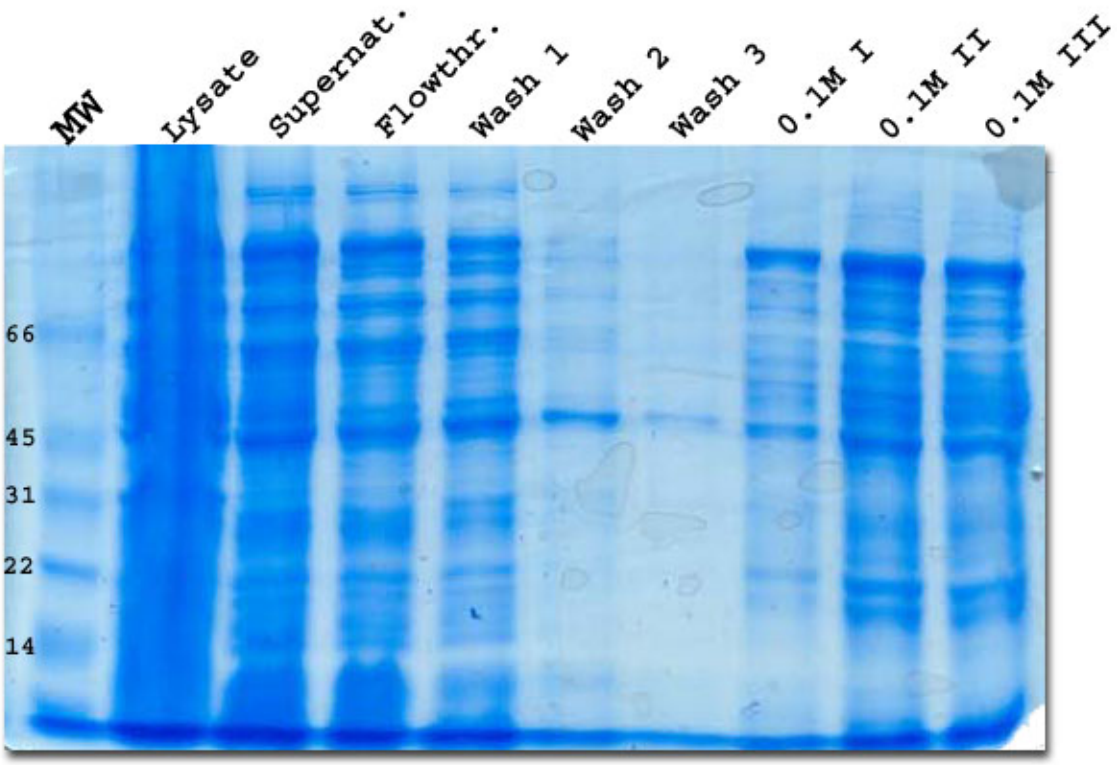

Figure 4: SDS-PAGE of *wt* 6PGDH fractions (1)

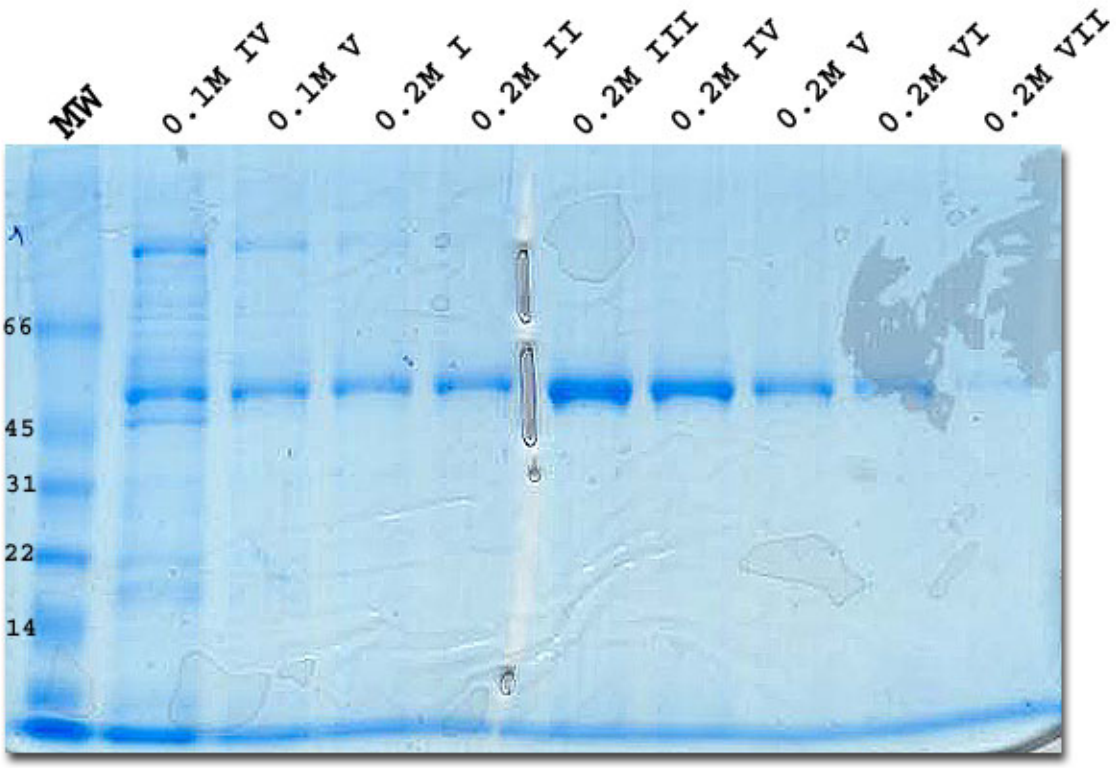

Figure 5: SDS-PAGE of *wt* 6PGDH fractions (2)

Step	Procedure	Volume	Units	Total Units	Protein	Tot. Protein	Spec. Activity	Yield	Purification
		[ml]	[U/ml]		[mg/ml]		[U/mg]		[*X]
1	Lysate	12.0	2.5966	31.1592	2.2550	27.0598	1.1515	100.0%	1.00
2	Centrifugation	12.0	2.4383	29.2596	2.2694	27.2327	1.0744	93.9%	0.93
3	Ni-NTA (pooled)	3.0	2.7050	8.1150	0.2694	0.8082	10.0408	26.0%	8.72

Table 1: Purification table for the *wt* enzyme

Discussion

Overall the purification is a good one for the enzyme. The final product is very clean and as one can see for the wt 6PGDH it does not harm the enzyme. Also it is very fast and easy to perform, which gives the researcher the opportunity o focus on the more important steps of his/her work.

After performing the purification again, we can assume that the lack of activity for the mutant enzyme has nothing to do with the used one-step purification. Due to the lack of activity it was not possible to make a purification table for this purification.

The purification of the *wt* enzyme was quite successful, only the unspecific elution of the 6PGDH was disappointing. The reason for this is most probably the method of regeneration. Till now we used acetic acid to regenerate the resin, but the new revision of the manual for the resin from Qiagen suggested the use of NaOH instead of acetic acid [6]. This may have caused degradation or a loss of metal ions in the resin or similar disturbance affecting the affinity of the purification step. In future purifications the old method will be used again.

References

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