Crystallographic studies on the binding of coenzyme analogs to D-glyceraldehyde-3-phosphate dehydrogenase from *Palinurus versicolor*

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Abstract In contrast with the coezyme, two coenzyme analogs, ADP-ribose and SNAD, bind non-cooperatively to D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Palinurus versicolor* (PV) GAPDH complexed with ADP-ribose and SNAD has been crystallized by the method of sitting-drop vapor diffusion. X-ray diffraction data analysis reveals that both crystals belong to the same space group (*C*2), and have similar cell dimensions: a =152.80 Å, b =100.35 Å, c =128.31 Å, β =110.28° and a =153.41 Å, b =100.51 Å, c =128.44 Å, β =110.48°, respectively. It is estimated that the asymmetric unit in each crystal contains 4 subunits. This is a novel crystal form which is quite different from that previously reported for holo- and apo-GAPDH from the same source. The result suggests that the binding of the two coenzyme analogs to GAPDH may lead to some significant conformational changes, which are different from those induced by the coenzyme binding. The self-rotation function indicates that the tetramer of these two GAPDH complexes also has good 222 symmetry. The structural analysis and the comparison with holo- and apo-GAPDH may give a clue to the cooperative mechanism of the enzyme.

Keywords: **D-glyceraldehyde-3-phosphate dehydrogenase, ADP-ribose, SNAD, crystal growth, X-ray analysis.**

The glycolytic D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH: EC 1.2.1.12) is a tetrameric enzyme which catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to form 1,3-diphosphoglycerate in the presence of NAD^+ and inorganic phosphate. GAPDH has four chemically identical subunits and the molecular weight is about 145 ku. The most interesting thing is that GAPDH shows various cooperative properties for binding NAD^+ and exhibits half-of-the-sites property[1] for reacting with certain acylating or ackylating reagents. There are two models (ligand-induced and pre-existing asymmetry) to explain the cooperative mechanism. Up to now, the high-resolution structures of apo-GAPDH and holo-GAPDH from different species have shown that the four subunits of GAPDH have good 222 symmetry[2—5] and significant conformational changes are observed during the apo-holo transition[6], thus supporting the ligand-induced asymmetry model. However, do these conformational changes relate closely to the cooperativity? How do the conformational changes transmit between the subunits? These questions have not been answered yet.

The biochemical studies of NAD^+ analog binding to GAPDH have provided much information for clarifying the cooperativity mechanism of the enzyme[7,8], but deep insight into the mechanism is restricted due to the limited structural information available. ADP-ribose is a potent inhibitor competitive with NAD^+; SNAD is effective as substrate to replace NAD^+ molecule and its complex with GAPDH has a small dissociation constant[7]. Therefore, the two coenzyme analogs were chosen because we were sure that they would strongly bind to the GAPDH. The more important is that they bind non-cooperatively to GAPDH, thus, the structure determination of the two complexes (ADR-GAPDH and SNAD-GAPDH) and the comparison to NAD^+-GAPDH may reveal differences between the conformational changes induced by the binding of non-cooperative coenzyme analogs and cooperative coenzyme to GAPDH. This may give some new clues to understanding the cooperativity of the enzyme.

1 Methods and results

(i) Purification and crystallization. PV holo-GAPDH was purified from the tail muscle of lobster in Chinese South Sea, *Palinurus versicolor*, according to the method of ammonium sulfate

fractionation[9]. Six recrystallizations were carried out and the specific activity was 150—180 unit/mg. The enzyme showed a single band on SDS-PAGE. It was then stored as a crystalline suspension in 76% saturated ammonium sulfate at 4 ℃. Apo enzyme was obtained by charcoal treatment of holo-GAPDH[10]. The specific activity was similar to holo-GAPDH and the ratio A_{280}/A_{260} equaled 1.8—2.0. The crystals of apo-GAPDH were then soaked into the solution containing 0.2 mmol/L ADP-ribose or SNAD and it was observed that the crystals would crack in 24 h. The result indicated that the two coenzyme analogs could strongly bind to GAPDH.

The crystals of ADR-GAPDH and SNAD-GAPDH were grown by the sitting-drop vapor diffusion in 20 μL inner-drop containing 5 mg/mL apo-GAPDH, 1 mmol/L EDTA, 1.6 mol/L $(NH_4)_2SO_4$, 1.75 mmol/L ADP-ribose or 2.0 mmol/L SNAD, 0.2 mol/L potassium phosphate buffer (pH 6.2) and 6 mL outer-drop containing 2.7 mol/L $(NH_4)_2SO_4$, 0.2 mol/L potassium phosphate buffer (pH 6.2). This vapor diffusion system was put into the constant-temperature box (18℃). Crystals would appear in 4 d and be harvested in two weeks (fig. 1). Twin crystals frequently appeared during crystallization. Single crystal was selected in subsequent crystallographic studies. Another crystal form of ADR-GAPDH was gotten by the hanging-drop technique. Droplets containing protein at a concentration of 5 mg/mL, 10% PEG4000, 5% 2-propanol and 10 mmol/L HEPES (pH 7.5) were equilibrated over wells containing 0.5 mL 20% PEG4000, 10% 2-propanol and 10 mmol/L HEPES (pH 7.5). The crystal showed rectangle shape. Further X-ray characterization was not carried out due to its weak diffraction ability.

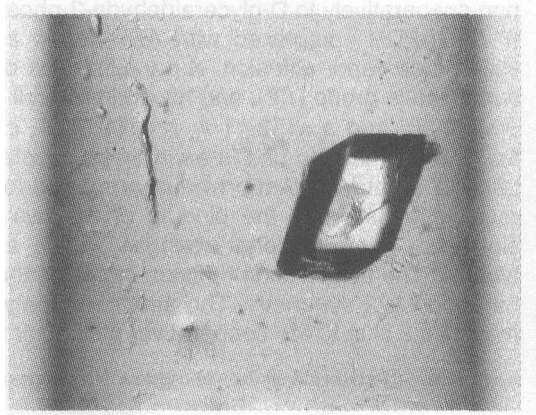

Fig. 1. Crystal of GAPDH complexed with ADP-ribose.

(ii) X-ray analysis. The data were recorded at 291 K at CuKα X-ray source using Mar Research Image Plate 345 system with a distance of 200 mm between the crystal center and detector, oscillation angle of 1°, the exposure time of 10 min for each frame and the collection range of 0°—180°. ADR-GAPDH crystal could diffract to 3.0 Å and SNAD-GAPDH to 2.8 Å. The data were processed using the software, Denzo and Scalepack[11]. Data analysis showed that the space group is *C*2. It was estimated that there were four subunits per asymmetric unit and the solvent occupied about 55% of the crystal volume [12]. The detailed crystallographic parameters are summarized in table 1.

Table 1 Crystallographic parameters and diffraction data of the two GAPDH complexes

	ADR-GAPDH	SNAD-GAPDH
Space group	*C*2	*C*2
Cell parameters	a = 152.80 Å	a = 153.41 Å
	b = 100.35 Å	b = 100.51 Å
	c = 128.31 Å	c = 128.44 Å
	β = 110.28°	β = 110.48°
No. of molecule per asymmetric unit	1	1
No. of observed reflections	92 755	119 278
No. of unique reflections	35 188	44 055
Completeness of data (%)	97.2	98.0
Completeness in the outest shell (%)	98.5	97.9
Resolution (Å)	3.0	2.8
R_{merge} (%)	13.8	12.6
R_{merge} in the outest shell (%)	35.0	37.9

apo-GAPDH has space group *C*2 and cell parameters of a=128.6 Å, b=99.8 Å, c=80.8Å, β=114.8°[13]. The crystal of holo-GAPDH is isomorphous with that of apo-GAPDH.

The self-rotation function was calculated by the program PLOARRFN in the soft package CCP4[14]

with an integration radius of 30 Å in the rotation angle range of $0 \leqslant \alpha \leqslant \pi$, $0 \leqslant \beta \leqslant \pi$ and $0 \leqslant \gamma \leqslant \pi$ and the angle bin of 5° and at two resolution ranges (12—5Å and 8—5Å). The results of ADR-GAPDH and SNAD-GAPDH were similar for the two resolution ranges. Only those results of SNAD-GAPDH within the resolution range 8—5Å were listed in table 2. Two prominent peaks (peaks II and III in table 2) were found. They correspond with two non-crystallographic axes from χ=180° and are perpendicular to each other from $\Delta\omega$ =90° and located in *ac* plane from φ =0°. Peak I (original peak) relates to the crystallographic twofold axis and is obviously perpendicular to the two non-crystallographic axes. Thus, it can be inferred that the tetramers of ADR-GAPDH and SNAD- GAPDH have good 222 symmetry and the molecular axes (P.Q.R.)[15] are parallel to the three axes mentioned above (three kinds of the combination in total). The structure determination is in progress.

Table 2 Self-rotation function result for SNAD-GAPDH

Peak No.		I			II			III		
Peak location	Eulerian angle/(°)	θ1	θ2	θ3	θ1	θ2	θ3	θ1	θ2	θ3
		0	180	0	0	131	180	180	49	0
	Polar angle/(°)	ω	φ	χ	ω	φ	χ	ω	φ	χ
		90	90	180	65	0	180	155	0	180
Peak height		100			71.2			71.2		

Resolution range: 8—5 Å; maximum noise: 28.9. ω, Angle from pole; φ, angle around equator.

2 Discussion

The crystallization conditions of PV ADR-GAPDH and SNAD-GAPDH using ammonium sulfate as precipitant were similar to those of previously reported holo- and apo-GAPDH from the same source. However different crystal forms were obtained although both belong to the same space group, *C*2. The crystals of ADR-GAPDH and SNAD-GAPDH (*C*2 (II)) have cell dimensions larger than 100 Å for all the three crystallographic axes and the non-hydrogen protein atoms in the asymmetric unit are 10 024. The unit cell volume of *C*2 (II) is twice as large as that of holo- and apo-GAPDH (*C*2 (I)). Therefore, the diffraction ability of the crystal was reduced and only medium resolution data could be collected using the regular X-ray source. If stronger X-ray source such as synchrotron radiation is used, the resolution and quality of the data may be improved.

Structures of GAPDH from *Bacillus stearothermophilus* and *Palinurus vesicolor* indicated that the obvious conformational changes including the relative motion between the two domains[6] can be observed during the apo-holo transition. In comparison with NAD^+ molecule, the nicotinamide part is absent in ADP-ribose, and only one atom changed (from O to S) in the thionicotinamide part in SNAD. However they bind non-cooperatively to GAPDH. If the crystals of apo-GAPDH soaked into the solution containing ADP-ribose or SNAD, the crystals would crack. The result indicates that the binding of these two coenzyme analogs to GAPDH may lead to some significant conformational changes. The differences in cell parameters between *C*2(I) and *C*2(II) suggest that the conformational changes induced by these two coenzyme analogs may be different from the conformational changes induced by NAD^+ molecules, and the isomorphism of the crystals of the two complexes suggests the similarity in the conformational changes induced by these two coenzyme analogs. These similarities and differences may be the key point to understand the negative cooperativity. The detailed structural analysis and comparison of the two complexes may throw some lights on clarifying the negative cooperativity of the binding of NAD^+ molecule to GAPDH.

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Transformation of plant young proembryos by electroporation

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Abstract It is first reported that plant young proembryos expressed exogenous reporter genes by electroporation. Young proembryos with 8—32 cells and globular proembryos with 250—400 cells could be isolated by enzymatic maceration combined with microdissection. After electroporation with *GUS* or *GFP* genes, the proembryos were cultured for 1—2 d in KM8p medium. At the field strength of electroporation 500—1 500 V/cm, blue reaction of GUS or green fluorescence of GFP could be observed in the proembryos. The highest transient expression frequency of young proembryos (2.2%) was obtained at the field strength of 750 V/cm, whereas the highest frequency of globular proembryos (5.9%) was obtained at the field strength of 1 250 V/cm. Taking the proportion of transformed cells in the whole cells of proembryos as efficient transformation frequency, the efficient transformation frequency of the young proembryos was 7 times that of the globular proembryos.

Keywords: electroporation, GUS, GFP, proembryo, *Nicotiana tabacum*.

Electroporation is an efficient way for direct gene transfer in plants. Transient or stable expression of exogenous genes have been obtained in suspended cells[1], protoplasts[2,3], tissue[4] and pollen[5,6]. However, regenerated plants derived from these materials often showed various abnormalities and reduced fertility due to difficulty in the mutation and regeneration caused by prolonged culture. Recently, plant embryo has been taken as an ideal material for transformation because of its normal development and strong regeneration ability. Using electroporation, Topfer et al.[7] first electroprated exogenous gene into wheat immature embryos and observed its transient expression. Subsequently, transient or stable expressions were obtained by electroporation in immature or mature embryos of rice[8], maize[9—11] and wheat[12]. Thus far these researchers are limited to mature and immature embryos.