53. On the Cofactor of Glucose Dehydrogenase of *Pseudomonas fluorescens*^{*)}

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Hauge¹⁾ has reported on the purification of glucose dehydrogenase (EC 1.1.99.a) obtained from a soluble fraction of *Bacterium anitratum*. The enzyme seemed to be also present in a particle-bound state, and it was found to possess a novel prosthetic group (cofactor) showing absorption maxima at 248 and 330 nm. The cofactor could be released from the enzyme by gel filtration at low pH, and the apoenzyme obtained was reactivated by addition of a cofactor preparation (supernatant obtained from the holoenzyme after boiling). According to Niederpruem and Doudoroff²⁾ the aldose dehydrogenase of *Rhodopseudomonas spheroides* exists in the apo-form both in the soluble and particulate fraction of bacterial cells, and the cofactor is released from the particulate fraction by heating or treating the fraction with salt at high concentrations. They also found that a particulate fraction could be reactivated by the addition of a boiled extract of *B. anitratum*. Imanaga *et al.*³⁾ once suggested the participation of FAD and some metal ion in the dehydrogenation of GlcN by a digitonin-extract obtained from the particulate fraction of *Pseudomonas fluorescens*, but later^{**)} they could not detect the participation of FAD and several other compounds in the reaction. In their studies on the metabolism of GlcN, the substrate used for testing the activity of dehydrogenase was mainly GlcN. Subsequent experiments indicated, however, that the glucose dehydrogenase of *Ps. fluorescens*⁴⁾ also acts upon GlcN.

In this paper are reported the procedures of obtaining from a DOCh-extract of the particulate fraction of *Ps. fluorescens* the apo-glucose dehydrogenase freed from its specific cofactor and the method

^{*)} Abbreviations used: DCPIP for 2,6-dichlorophenolindophenol, DOCh for deoxycholate, EDTA for ethylene diaminetetra-acetate, FAD for flavin adenine dinucleotide, FMN for flavin mononucleotide, Glc for D-glucose, GlcN for D-glucosamine, NAD⁺ for nicotinamide adenine dinucleotide, NADP⁺ for nicotinamide adenine dinucleotide phosphate, TLC for thin layer chromatography.

^{**)} Reported at the 19th Symposium on Enzyme Chemistry in 1968 (Kanazawa Univ., Kanazawa).

of purifying the latter which was found to be accumulated in the culture medium as a metabolic product of the bacterium.*)

Experiments and results. The enzyme activity was assayed by measuring the reduction in absorbance at 600 nm (ΔA_{600}) of DCPIP in 5 min at 20°C, using the cuvettes of 1-cm light path.

Assay of the apoenzyme—The complete mixture in the cuvette in a total volume of 3.5 ml consisted of: enzyme solution, 10^{-3} M CaCl_2 , 10^{-3} M KCN, cofactor preparation (the supernatant of boiled holoenzyme) in excess of the amount required for the apoenzyme, 0.25 μmol of DCPIP, 15 μmol of substrate (GlcN or Glc) and 2.5×10^{-2} M veronal-HCl buffer of pH 7.0 (buffer). The substrate was omitted from the reference cuvette. The apoenzyme was incubated in the buffer solution with Ca^{2+} for 2 min, and then with the cofactor preparation for 10 min to reconstitute the holoenzyme. The reaction was started by adding the substrate. The amount of apoenzyme was calculated by subtracting the amount of holoenzyme remaining in the enzyme preparation from the total amount of the enzyme.

Assay of the holoenzyme—Assay was carried out as described above, the only difference being the omission of the step of addition of the cofactor.

Assay of the cofactor—The cofactor was assayed in the same way as that used for the apoenzyme, except that the apoenzyme (“15–25% $(\text{NH}_4)_2\text{SO}_4$ fraction”) was added in excess of the cofactor, and that the DCPIP solution was used as a reference. The amount of the cofactor was calculated by subtracting the ΔA_{600} -value corresponding to the amount of holoenzyme remaining in the apoenzyme preparation.

Determination of the amount of protein. The amount of protein was determined by the method of Lowry *et al.*,⁵⁾ crystalline albumin of bovine plasma being used as the standard.

Preparation of the apoenzyme. *Ps. fluorescens* was cultivated in the medium described previously.⁶⁾ The washed cells were suspended in 5×10^{-3} M buffer (packed cells: buffer=1:1 by vol.), and sonicated (9 kHz) for 10 min. The sonicated preparation was centrifuged at $1.6 \times 10^4 \times g$ for 20 min, and the loose upper part of the precipitate was mixed with 2% DOCh-Na solution (precipitate gruel: DOCh=1:1 by vol.), pH 7.0, and kept overnight in an ice box. Then the mixture was centrifuged at $10^5 \times g$ for 40 min. The supernatant (DOCh-extract) was slowly mixed with 10^{-1} M CaCl_2 solution (supernatant: CaCl_2 =3:1 by vol.) and the mixture was centrifuged. The supernatant (crude enzyme) was mixed with 10^{-1} M EDTA, keeping

*) Reported at the 44th and 51st Annual Meeting of the Japanese Biochemical Society in 1971 (Tohoku College of Technology, Sendai) and 1978 (Kyoto Kaikan, Kyoto).

the pH at 7.0 with 10^{-1} M NaOH. After the decrease in pH had stopped, 10^{-2} M excess of EDTA was added and the mixture was then dialyzed against 5×10^{-3} M buffer (EDTA-enzyme). Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the solution at pH 7.0. The precipitate (15–25% $(\text{NH}_4)_2\text{SO}_4$ fraction) obtained in the second fractionation of the first 15–45%-fraction was dissolved in 5×10^{-3} M buffer and dialyzed for 24 h against 5×10^{-4} M buffer. The whole process was carried out at the temperatures below 10°C . Table I summarizes the results of the process. All the compounds examined, namely FAD, FMN, NAD^+ , NADP^+ , etc., could not, unlike the cofactor preparation described in this paper, reactivate the apoenzyme. EDTA-treatment removed the essential metal ion from the holoenzyme and the unidentified cofactor was released during subsequent dialysis. The final preparations were found to contain the holoenzyme in amounts that were usually less than 10% of the total enzyme present in them. The apoenzyme preparations were stable on storage in freezer and was used for the assay of the cofactor in our studies described below. These preparations exhibited dehydrogenase activity toward Glc with a strength about 4 times that toward GlcN.

Table I. Preparation of apoenzyme

Enzyme preparation	Total volume (ml)	Total protein (mg)	Total activity Holoenzyme activity (ΔA_{600})	Specific activity ($\Delta A_{600}/\text{mg}$)	Yield (%)
DOCh-extract	80	1504	760	0.50	
			400		
Crude enzyme	193	955	724	0.76	95.3
			417		
EDTA-enzyme	278	856	639	0.75	84.1
			189		
15–45%* $(\text{NH}_4)_2\text{SO}_4$ fraction	40	296	656	2.2	86.3
			34		
15–25%* $(\text{NH}_4)_2\text{SO}_4$ fraction	30	92	630	6.8	82.9
			27		

* $100 \times$ amount (g) of the salt added to the solution/maximum amount (g) of the salt soluble in the same volume of water.

Accumulation of the cofactor in culture medium. *Ps. fluorescens* was grown for 2–4 days at 27°C with aeration in the dark. The asparagine-glycerol medium⁷⁾ (1/10 concentration) was used with some modification of inorganic components.⁸⁾ The amount of cofactor (substrate: Glc) in 18 liter-culture reached ca. 9×10^5 (ΔA_{600}) in 120 h (growth of bacterium as measured turbidimetrically at 600 nm: 1.6),

while $\text{ca. } 10^4$ (ΔA_{600}) was usually present in the boiled extract of the holoenzyme derived from $\text{ca. } 80$ g (wet weight) of packed cells.

Purification of the cofactor. The supernatant (showing greenish-yellow fluorescence) obtained by centrifugation of the culture was treated with charcoal (36 g). The charcoal was removed by filtration and washed first with water and then with 50% ethanol. The active principle was eluted with 10% pyridine and the eluate was concentrated under reduced pressure at 37°C . The concentrated sample was adjusted to pH 7.0 and applied to a DEAE-cellulose column ($\text{ca. } 3 \times 11$ cm; fraction volume: $\text{ca. } 10$ ml) which had been equilibrated with 0.5 M CH_3COONa , pH 7.0. The column was eluted stepwise with 0.5, 0.75 and 1.0 M acetate, pH 7.0. Fractions containing the active principle (1.0 M acetate; recovery: 93%) were combined and again treated with charcoal. The eluate (20% pyridine; average recovery in the two steps: 63%) was concentrated and applied to several sheets of filter paper (Whatman No. 3MM) as a 1×40 cm streak. Chromatograms were developed with n-butanol-ethanol-water (4:3:5 by vol.) and the reddish-brown band of R_f 0.38 (fluorescence: yellow when wet and light-blue when dry on excitation at 365 nm) was eluted with water. The combined eluate (recovery: 92%) was concentrated, applied to TLC-plates (Merck: silica-gel aluminum plate) and developed with the same solvent system. The reddish-brown band with light-blue fluorescence was eluted with water. The final preparation gave a single spot on TLC in three solvent-systems (n-propanol-water-conc. $\text{NH}_3\text{aq.}$, 40:20:1; methanol-n-butanol-benzene-water, 2:1:1:1; and the same system described above).

Spectral properties of purified cofactor. 1) Fig. 1a shows the absorption spectra of the purified cofactor. These spectra coincided well with that reported for the prosthetic group of glucose dehydrogenase of *B. anitratum*¹⁾ and that of alcohol dehydrogenase (EC

Fig. 1. a: Absorption spectra of the cofactor in different solvents. (—), H_2O ; (---), $\text{ca. } 0.1$ M NaOH ; (·····), $\text{ca. } 0.1$ M HCl . b: Fluorescence spectra of the cofactor in different solvents. Lines indicate the same solvents as given in Fig. 1a. The arrow ($\uparrow$) indicates the increase of sensitivity of the spectrophotofluorometer in scanning the weak emission of alkaline solution of the cofactor. Excitation at 365 nm.

1.1.99.8) of *Ps. sp.* M27.⁹⁾ 2) Fig. 1b shows the fluorescence spectra of the purified cofactor on excitation at 365 nm. 3) A solution of cofactor (neutral, N₂-bubbled) was mixed with a trace of solid NaBH₄. A characteristic change (appearance of a new peak at 303 nm) was observed and then the original spectrum was gradually recovered completely in 5–10 min (Fig. 2). Hauge¹⁾ has reported a very similar observation using a boiled extract of purified glucose dehydrogenase of *B. anitratum*. The recovered compound showed the same behavior on TLC and the same cofactor activity as the original material. These results show that the reduction-autoxidation of the cofactor is a completely reversible process.

Fig. 2. Change of absorption spectra of the cofactor caused by treatment with borohydride. 1, untreated; 2, immediately after addition of NaBH₄; 3, several minutes after addition of NaBH₄. In all cases, scanning was carried out within about 3.5 min.

It has been reported by several workers that there are glucose dehydrogenases having cofactors that are different from NAD⁺, NADP⁺, FMN or FAD.^{1),2),4),10)} According to Hauge the cofactor of a dehydrogenase from *B. anitratum* seemed to be, judging mainly from its spectroscopic properties, a substituted naphthoquinone, while Anthony and Zatman⁹⁾ first reported that *Ps. sp.* M27 contains an alcohol dehydrogenase whose cofactor showed absorption and fluorescence spectra resembling that of a pteridine derivative to be formed as a degradation product of tetrahydrofolic acid. These workers could, however, not definitely determine the chemical nature of those cofactors, presumably because of the insufficiency of the samples obtained in their experiments. Using the cofactor we obtained from the culture medium of *Ps. fluorescens*, we observed that on treating it with borohydride there appeared an absorption peak at about 300 nm which seemed to suggest the formation of 5,6,7,8-tetrahydropterin, a substance known to be formed by reduction of 7,8-dihydropterin (Fig. 3).^{11)–13)} Several species of *Pseudomonas* have been reported to produce pteridine derivatives in cultivation media.^{7),14),15)} It may not be presumptuous to assume that the cofactor we obtained might be identical or at least closely related to those studied by Hauge as well

Fig. 3. Structures: (I), pterins; (II), 7,8-dihydropterins; (III), quinonoid form of (II); (IV), 5,6,7,8-tetrahydropterins.

as by Anthony and Zatman. Further studies on the chemical nature of the cofactor in question is now in progress.

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