Simple Purification and Improvement of Stability of Alcohol Oxidase from a Methanol Yeast, *Candida boidinii* S2, by Forming an Enzyme-Azide Complex

Yasuyoshi SAKAI* and Yoshiki TANI

Research Center for Cell and Tissue Culture and *Department of Agricultural Chemistry,
Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

Received August 21, 1987

It was found that azide bound to alcohol oxidase non-covalently and caused a color change from yellow to red. Alcohol oxidase was purified with a high yield from methanol-limited chemostat-grown cells of *Candida boidinii* S2 as an enzyme-azide complex by a simple procedure. That is, a cell-free extract was prepared from cells treated with a cationic detergent, Cation M2, and alcohol oxidase amounted to more than 80% of the soluble protein. Inactivation of the enzyme by H_2O_2 and aldehydes was decreased in the complex. The results were discussed as to the production of various aldehydes with the yeast cells.

In methanol yeasts, methanol is oxidized to formaldehyde in peroxisomes by alcohol oxidase (EC 1.1.3.13).¹⁾ Methanol-grown cells contain large amounts of alcohol oxidase under appropriate culture conditions.^{2,3)} Previously, we established high-yield reaction systems for the production of formaldehyde and other useful aldehydes. In the course of our investigation, we found that cells which were heat-treated in the presence of NaN_3 changed in color to red and showed higher formaldehyde productivity than the intact cells.⁴⁾ Great differences in molar yields between aldehydes were also observed.⁵⁾ On the other hand, we reported that cells treated with a cationic detergent, Cation M2, exhibited the most efficient formaldehyde production.⁶⁾ During this treatment, methanol-dissimilating enzymes other than alcohol oxidase were found to leak out from cells.

In this study, the effect of the azide anion on the native alcohol oxidase was first studied. A simple purification procedure for the enzyme was then established, involving the formation of an enzyme-azide complex after Cation M2 treatment of cells. Several features of the native and azide-complex enzymes

were compared and discussed from the viewpoints of aldehyde production and enzyme inactivation.

MATERIALS AND METHODS

Materials. The cationic detergent used, cetyldimethylbenzylammonium chloride (Cation M2), was a product of Nippon Oil & Fats Co., Ltd., Tokyo. The pure formaldehyde solution was prepared from paraformaldehyde (Nakarai Chemicals Co., Ltd., Kyoto). All other chemicals were purchased or prepared as described previously.⁷⁾

Organism and cultivation. Strain AOU-1 derived from *Candida boidinii* S2⁷⁾ was chemostat-grown on methanol at the dilution rate of 0.075 hr^{-1} as described previously.³⁾

Assay of alcohol oxidase. The activity of alcohol oxidase was determined by following the formation of H_2O_2 as described previously (the ABTS/POD method).⁷⁾

Alternatively, the enzyme activity was measured by determining the amount of formaldehyde formed (the HCHO method): the reaction mixture contained $4.8\text{ }\mu\text{mol}$ methanol, $39.6\text{ }\mu\text{mol}$ potassium phosphate buffer, pH 7.5, and the enzyme ($1.0\sim 2.5\text{ }\mu\text{g}$), in a final volume of 1.2 ml. Incubation was carried out for 10 min at 28°C . Formaldehyde was assayed according to the method of Nash.⁸⁾ One unit (U) of the enzyme was defined as the amount of enzyme forming $1\text{ }\mu\text{mol}$ of formaldehyde per min.

Prior to starting the reaction, the reaction mixture was

bubbled with air vigorously for 2 min for both methods.

Purification of alcohol oxidase. All purification procedures were performed at 4~6°C.

a) *Native enzyme.* Chemostat-grown cells were disrupted with a Kaijo-Denki ultrasonic oscillator at 19 kHz for 40 min. The purification procedures were essentially according to Tani *et al.*⁹⁾ except that DEAE-Sephacel was used instead of DEAE-Cellulose. The purified native enzyme showed specific activities of 7.8~8.0 U/mg protein with the ABTS/POD method.

b) *Enzyme-azide complex.*

1. Cation M2-treatment of cells. Cells were washed with 0.1 M potassium phosphate buffer, pH 7.5, and then suspended in the buffer containing 0.1% Cation M2 to a concentration of 30 mg dry cell weight/ml. The suspension was incubated at 37°C for 8 hr under static conditions. Then the Cation M2 was removed and the cells were washed with the buffer three times by centrifugation at $10,000 \times g$.

2. Preparation of a cell-free extract. Cation M2-treated cells were disrupted by sonic oscillation as described above, and then NaN_3 was added to a final concentration of 3 mM. With the addition of NaN_3 , the color of the cell-free extract changed from yellow to red. A 10% solution of streptomycin sulfate was added to the crude extract with constant stirring to give a final concentration of 1%. The resulting precipitate was removed by centrifugation at $10,000 \times g$ for 20 min.

3. Ammonium sulfate precipitation. The obtained supernatant was fractionated by ammonium sulfate saturation (50~60%).

4. Sephadex G-200. The concentrated enzyme solution from Step 3 was applied to a Sephadex G-200 column (1.5 $\times$ 80 cm) equilibrated with 30 mM potassium phosphate buffer, pH 7.5, containing 3 mM NaN_3 . The purified alcohol oxidase-azide complex showed a specific activity of 8.2 U/mg protein with the ABTS/POD method.

Analytical methods. Protein was determined by the method of Bradford¹⁰⁾ with a protein assay kit (Bio Rad Laboratories). Electrophoresis was carried out in a 12% polyacrylamide slab gel by the method of King and Laemmli¹¹⁾ as described previously.⁷⁾ Spectrophotometric analysis was performed with a Hitachi 220A double beam spectrophotometer.

RESULTS

Effects of azide on native alcohol oxidase

a) *Changes in absorption spectra and azide binding.* The purified native enzyme showed two absorption maxima at 380 nm (with a shoulder at 394 nm) and 455 nm. When NaN_3 or $\text{Ba}(\text{N}_3)_2$ was added to the enzyme solution,

the absorption spectrum changed (Fig. 1). The peak at 380 nm blue-shifted to 368 nm and that at 455 nm red-shifted to 483 nm. The increase in the range of 520~525 nm could cause the color change from yellow to red. Thus, the color change of NaN_3 -treated cells was proved to be due to that of alcohol oxidase.

FIG. 1. Adsorption Spectra of the Alcohol Oxidase in the Presence and Absence of Azide.

Alcohol oxidase (3.24 mg/ml) was dissolved in 30 mM potassium phosphate buffer, pH 7.5, (—), or the enzyme dissolved in the same buffer containing 2.5 mM NaN_3 (-----).

FIG. 2. Azide as a Competitive Inhibitor of Alcohol Oxidase.

Azide was added as the sodium (●, ○) or barium salt (▲, △). The activity of the enzyme was assayed by the ABTS/POD method, as described in MATERIALS AND METHODS. The substrate, methanol, concentration in the assay mixture was 0.4 mM (●, ▲) or 1.0 mM (○, △).

The red color of the enzyme was recovered on ammonium sulfate precipitation. However, the red color turned the original yellow color on passage through Sephadex G-200 gel or extensive dialysis against azide-free buffer. This shows that the spectral change was due to the non-covalent binding of azide to the enzyme.

b) *Azide as a competitive inhibitor.* The enzyme activity was strongly inhibited by NaN_3 or $\text{Ba}(\text{N}_3)_2$. From a Dixon plot with various concentrations of NaN_3 or $\text{Ba}(\text{N}_3)_2$, the K_i value for azide was estimated to be 0.015 mM (Fig. 2). The inhibition was competitive with respect to methanol. The K_m value for methanol of the native enzyme was determined to be 0.53 mM from a Lineweaver–

Burk plot.

c) *Azide as a stabilizer of alcohol oxidase.* The native enzyme gradually lost its activity during storage. However, this inactivation could be partially repressed by the addition of azide. As shown in Table I, 93% of the initial activity remained after 1-day incubation at 30°C in the presence of 2.5 mM NaN_3 , whereas less than half the activity remained without NaN_3 . At 4°C, the enzyme did not lose its activity at all in the presence of 2.5 mM NaN_3 for at least 4 days.

Spontaneous release of formaldehyde from native alcohol oxidase

When alcohol oxidase was kept at 4°C for several days, H_2O_2 was detected in the solution without the addition of substrate. As shown in Table II, formaldehyde was also released from the native enzyme during storage. These facts suggest that H_2O_2 was produced on the oxidation of the released formaldehyde to formate,

TABLE I. AZIDE AS A STABILIZER FOR ALCOHOL OXIDASE

The freshly prepared enzyme (3.24 mg/ml) was dissolved in 30 mM potassium phosphate buffer, pH 7.5, containing the indicated concentrations of azide salts, and then stored at 4°C or 30°C. After incubation for 1–2 days, the enzyme activity was assayed by the HCHO method. The remaining activity (%) is expressed as the ratio as to the initial activity.

Incubation (days)		Remaining activity (%)			
		NaN ₃		Ba(N ₃) ₂	None
		0.25 mM	2.5 mM	1.25 mM	
4°C	1	102	103	99	86
	2	102	101	104	71
30°C	1	61	93	86	44
	2	53	94	74	41

TABLE II. THE SPONTANEOUS RELEASE OF FORMALDEHYDE FROM ALCOHOL OXIDASE DURING STORAGE

The freshly prepared native enzyme (3.24 mg/ml) was dissolved in 30 mM potassium phosphate buffer, pH 7.5, at 4°C. After several days, the formaldehyde concentration in the solution was determined.

Incubation (days)	Formaldehyde (mM)
0	0.3
3	1.4
7	2.2

TABLE III. SIMPLE PURIFICATION OF ALCOHOL OXIDASE AS AN ENZYME-AZIDE COMPLEX FROM CATION M2-TREATED CELLS OF *C. boidinii* S2 STRAIN AOU-1

The purification procedures are described in MATERIALS AND METHODS. The enzyme activity was determined by the ABTS/POD method.

		Total protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Yield (%)
I	Cell-free extract	226	6.8	1537	100
II	Ammonium sulfate (50~60%)	103	7.8	803	52
III	Sephadex G-200	83	8.2	681	44

and that the enzyme was gradually inactivated by the released formaldehyde or H_2O_2 . The stabilization of the enzyme by azide described above would be due to inhibition of the autonomous formaldehyde oxidation.

In the enzyme solution (3.24 mg protein/ml), the formaldehyde concentration reached as high as 2.2 mM, after 7 days. Assuming that the molecular weight of the enzyme subunit is 72,000,⁷⁾ about 46 molecules of formaldehyde was calculated to be released from one enzyme subunit. The content of formaldehyde per enzyme subunit was lower in batch-cultured cells than in methanol-limited chemostat-grown cells.

FIG. 3. SDS-Polyacrylamide Electrophoretic Analysis of Cell-free Extracts from Heat- or Cation M2-treated Cells, and Intact Cells.

The conditions are given under MATERIALS AND METHODS. Cell-free extracts from: lane 1, intact cells; lane 2, heat-treated cells; lane 3, 2 hr Cation M2-treated cells; lane 4, 8 hr Cation M2-treated cells; lane 5, the alcohol oxidase-azide complex, purified as in MATERIALS AND METHODS; lane 6, marker proteins: phosphorylase *b* (M_r = 94,000), bovine serum albumin (67,000), ovalbumin (43,000), bovine erythrocyte carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α -lactalbumin (14,000).

Simple purification of alcohol oxidase as an enzyme-azide complex using Cation M2 treatment

Methanol-dissimilating enzymes other than alcohol oxidase were found to leak from cells on Cation M2 treatment.⁶⁾ On treatment with 0.1% Cation M2 for 8 hr, alcohol oxidase remained in the cells. A higher Cation M2 concentration, lower cell concentration or longer incubation time caused the leakage of alcohol oxidase from cells.

As summarized in Table III, the purification of alcohol oxidase was simply achieved from Cation M2-treated cells, in the form of an enzyme-azide complex, which prevented inactivation.

Figure 3 shows the SDS-polyacrylamide gel electrophoretic (SDS-PAGE) patterns of cell-free extracts of intact cells, and heat-treated and Cation M2-treated cells, and the purified alcohol oxidase-azide complex. Heat treatment was performed by incubating the cell suspensions at 37°C for 24 hr.⁴⁾ The protein pattern did not change on the heat treatment. On the other hand, cells gradually lost almost all polypeptides during the Cation M2 treatment. The only conspicuous exception was a 72 kDa protein, which corresponds to a subunit of alcohol oxidase, the intensity of the band of which remained unchanged. Thus, the leakage of proteins was not specific for formaldehyde and formate dehydrogenases, and catalase. The retention of proteins on Cation M2 treatment seemed to be rather specific for

TABLE IV. SUBSTRATE SPECIFICITY OF THE ALCOHOL OXIDASE-AZIDE COMPLEX

The substrate concentration was 4 mM in each reaction mixture. The enzyme activity was determined by the ABTS/POD method.

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Methanol	100	Formaldehyde	27
Ethanol	75	Acetaldehyde	5
<i>n</i> -Propanol	32	Propionaldehyde	0
Allyl alcohol	45	Acrolein	0

alcohol oxidase.

The specific activity of the cell-free extract from intact cells was 3.1 U/mg protein (with the ABTS/POD method). Thus, alcohol oxidase was purified 2.1-fold by the Cation M2 treatment. Comparison of the specific activities with the purified enzyme or densitometric analysis after SDS-PAGE showed that the cell-free extract from Cation M2-treated cells contained an amount of alcohol oxidase corresponding to more than 80% of the total soluble protein. From such an enzyme solution, the alcohol oxidase could be purified to homogeneity, as judged from the single band on SDS-PAGE, through only two steps, ammonium sulfate precipitation and Sephadex G-200 gel permeation chromatography.

Stability of the native enzyme and the enzyme-azide complex against inactivation by H₂O₂ and aldehydes

The substrate specificity of the enzyme-azide complex is shown in Table IV, which is indistinguishable from that of the native enzyme. Both enzymes showed activities against short-chain alcohols, formaldehyde and acetaldehyde. The inactivation by H₂O₂ and several aldehydes was compared between these enzymes in the presence or absence of catalase

(Table V). H₂O₂ was more toxic than aldehydes because longer incubation was necessary for inactivation by aldehydes than that by H₂O₂ at the same concentration (10 mM). Although acrolein was not a substrate, the enzyme was completely inactivated, irrespective of the presence of catalase. On the other hand, acetaldehyde did not inactivate the enzyme so much. These results showed that the inactivation by acrolein was not due to the formed H₂O₂ but to acrolein itself. In the case of inactivation by formaldehyde, the enzyme might be inactivated by both formaldehyde itself and the produced H₂O₂. Inactivation by acrolein was not complete in the enzyme-azide complex. The enzyme-azide complex also showed higher remaining activity than the native enzyme on inactivation by other agents. Inactivation by H₂O₂ was also repressed in the complex. Thus, the binding of azide to alcohol oxidase improved the stability against inactivation by both H₂O₂ and aldehydes.

DISCUSSION

It was found that azide was a competitive inhibitor as to methanol and the enzyme-azide complex showed improved resistivity against

TABLE V. PROTECTION OF ALCOHOL OXIDASE BY AZIDE AGAINST INACTIVATION BY H₂O₂ OR ALDEHYDES

The native enzyme or the alcohol oxidase-azide complex (2 µg) was dissolved in 1.12 ml of 0.1 M potassium phosphate buffer, containing the indicated concentrations of additives. Catalase was added to the concentration of 1 U/ml. The solution was then incubated at 30°C, for 20 min with the addition of H₂O₂ or for 24 hr with the addition of aldehydes. After the incubation, assay of the alcohol oxidase activity was started by the addition of methanol according to the HCHO method, as described in MATERIALS AND METHODS. The remaining activity (%) is expressed as the ratio as to the initial activity.

	Remaining activity (%)			
	Native enzyme		Enzyme-azide complex	
	- Catalase	+ Catalase	- Catalase	+ Catalase
1 mM H ₂ O ₂	42	95	85	98
10 mM H ₂ O ₂	5	75	43	90
10 mM Formaldehyde	25	36	55	72
10 mM Acetaldehyde	60	70	95	98
10 mM Acrolein	0	0	15	19

both aldehydes and H_2O_2 . Methanol was also reported to partially protect the enzyme from inactivation, since lower inactivation rates were observed at higher methanol concentrations.¹²⁾ These facts showed that azide bound around the catalytic site of alcohol oxidase and improved the formaldehyde productivity⁴⁾ through protection of the site against aldehydes and H_2O_2 . The oxidation of ethanol and 1-propanol was completely accomplished by cells of *C. boidinii* S2, but in the cases of methanol and allyl alcohol the substrates remained even when they were added at low concentration.⁵⁾ This incomplete oxidation would be mainly due to inactivation of the alcohol oxidase by these aldehydes. Although H_2O_2 is more inhibitory toward the purified enzyme than aldehydes, inactivation by formaldehyde or allyl alcohol rather than H_2O_2 would limit the molar yields of these aldehydes under the reaction conditions with catalase.

Recently, the spontaneous release of formaldehyde from alcohol oxidase was described by Hopkins and Muller.¹³⁾ They proposed the role of the formaldehyde adduct as a buffering system for formaldehyde *in vivo*. Under methanol-limited chemostat-conditions, cells contain much more alcohol oxidase than is needed,^{2,3)} and thus would tend to overproduce formaldehyde. The higher amount of the formaldehyde adduct in chemostat-grown cells than in batch cultured-cells would support the idea of "the formaldehyde buffer system" *in vivo*. The formaldehyde-adduct content was higher in alcohol oxidase from *C. boidinii* S2 (26 molecules/subunit) than that reported for the enzyme from *Pichia pastoris* (7.6 molecules/subunit).¹³⁾ This might indicate a higher buffering capacity of *C. boidinii* S2 and reflect its characteristics as a formaldehyde producer.⁷⁾ The formaldehyde-adduct enzyme might also be involved in the regulation of alcohol oxidase by glucose (catabolite inactivation)^{14,15)} through its affecting the proteolytic susceptibility of the enzyme.

The amount of released formaldehyde during storage was lower at 30°C than at 4°C.

Formaldehyde would be oxidized into formate at 30°C with the formation of H_2O_2 , which is much more toxic than formaldehyde. The inactivation of alcohol oxidase by both acrolein and H_2O_2 was repressed at 4°C (data not shown) as well as the formation of H_2O_2 . Thus, the optimum temperature for aldehyde production, 4°C,^{5,16)} was also due to reduction of the susceptibility of the enzyme to attacks by H_2O_2 and aldehydes.

The purification yield of the alcohol oxidase from methanol-limited chemostat cultures was usually low (unpublished data), probably due to the high content of the formaldehyde-adduct enzyme. The increased release of formaldehyde in the concentrated enzyme solution (data not shown) would cause inactivation of the enzyme, which may be the reason for the rather low yield after ammonium sulfate fractionation or DEAE-Sephacel column chromatography. However, the present simple purification method for alcohol oxidase, as an enzyme-azide complex, could minimize such inactivation, and successfully gave a good purification yield from methanol-limited chemostat-grown cells.

Alcohol oxidase from methanol yeasts, *Hansenula polymorpha* and *C. boidinii*, contains a flavin semiquinone, which is not required for the oxidation of methanol.¹⁷⁾ The existence of a flavin semiquinone in the native enzyme was characterized by the existence of absorption maxima, *ca.* 373 nm, with a shoulder around 400 nm, and 477 nm.¹⁸⁾ The enzyme-azide complex also seemed to contain a flavin semiquinone as the native enzyme did. Spectral changes due to azide were observed for alcohol oxidase of a white rot fungus, *Poria contigua*,¹⁹⁾ and also recently in the case of a methanol yeast, *P. pastoris*.¹³⁾ The possibility of both enzymes being ferroflavoproteins was examined, however, the iron content was lower than that needed for a unit molar ratio with the alcohol oxidase subunit. The exact mechanism of the spectral changes on azide binding remains to be investigated. The K_i value of the *Pichia* enzyme for azide was reported to be 0.006 mM,¹³⁾ which was lower than that of the

enzyme from *C. boidinii* S2. Furthermore, the *Pichia* enzyme did not release azide under the conditions used, under which the enzyme remained active, such as on dialysis or Sephadex-G200 gel filtration.¹³⁾ The *Candida* enzyme excels in that the native enzyme could be easily prepared after purification as an enzyme-azide complex.

Acknowledgment. We wish to thank Professor H. Yamada, Department of Agricultural Chemistry, Kyoto University, for the valuable discussion during this work.

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