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Tetrazolium-dye-linked alcohol dehydrogenase of the methylotrophic actinomycete *Amycolatopsis methanolica* is a three-component complex

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Tetrazolium-dye-linked alcohol dehydrogenase (TD-ADH) of *Amycolatopsis methanolica* could be resolved into three protein components, which have been purified. Each of the components has the ability to reconstitute TD-ADH activity when combined with the other two. Component 1 is identical to the previously characterized methanol:*N*,*N*′-dimethyl-4-nitrosoaniline oxidoreductase (MNO), a decameric protein with 50-kDa subunits, each carrying a tightly bound NADPH. Component 2 is a high molecular mass (> 640 kDa) protein with subunits of 44 kDa and 72 kDa, and which possesses a low tetrazolium-dye-linked NADH dehydrogenase activity. The protein contains a yellow chromophore of unknown identity. Component 3 is a low molecular mass (15 kDa) protein containing a 5′-deazaflavin and at least one other low-molecular-mass compound with properties similar, but not identical, to those of nicotinamide coenzymes. The results suggest that alcohol oxidation by the TD-ADH complex is carried out by component 1 (MNO), after which transfer of the reducing equivalents (mediated by component 3) occurs to component 2, which (*in vitro*) is linked to the tetrazolium dye. Fractionation of *A. methanolica* extracts showed that most of the 5′-deazaflavin was present in component 3. Other gram-positive bacteria having a TD-ADH complex also produced 5′-deazaflavin. It is concluded that oxidation of primary aliphatic alcohols by *A. methanolica*, and probably also by other gram-positive bacteria containing MNO or TD-ADH, proceeds via TD-ADH. The likeliness of 5′-deazaflavin participation in this process is discussed.

Keywords: dye-linked alcohol dehydrogenase; methanol:*N*,*N*′-dimethyl-4-nitrosoaniline oxidoreductase; 5′-deazaflavin; methanol; formaldehyde.

The enzymology of methanol oxidation in gram-positive bacteria is much more complicated than that in their gram-negative counterparts, where this conversion is catalysed by the pyrroloquinoline-quinone-containing methanol dehydrogenase. This complexity is illustrated by the diverse methanol-oxidizing activities described for the actinomycete *Amycolatopsis methanolica*. Kato et al. (1975) reported that extracts of methanol-grown cells exhibit phenazine methosulphate/2,6-dichloroindophenol (Cl_2Ind) methanol dehydrogenase activity. Subsequently, Duine et al. (1984) reported the presence of a methanol dehydrogenase (MeOH-DH) in a multienzyme complex, displaying a Cl_2Ind-dependent activity that was stimulated by NAD addition. However, both these assays were difficult to reproduce. It was only after the development of a reproducible assay (van Ophem et al., 1991) that further progress in these studies became possible. Typical features of the assay are that only a few tetrazolium dyes are active [e.g. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)], it is stimulated by high phosphate or sulphate concentrations, and the relationship between the amount of extract and activity is non-linear. The latter two points suggested also this tetrazolium-dye-linked alcohol dehydrogenase (TD-ADH) activity originates from a multienzyme complex.

TD-ADH activity was found in several other gram-positive bacteria (but not in the tested gram-negative ones), including non-methylotrophs grown on ethanol (van Ophem et al., 1991). A broad substrate specificity for primary alcohols was observed, suggesting that a hitherto undiscovered alcohol dehydrogenase might be involved in alcohol oxidation by gram-positive bacteria. Further studies resulted in identification and purification of two nicotinoprotein alcohol dehydrogenases in *A. methanolica*, one of which is methanol:*N*,*N*′-dimethyl-4-nitrosoaniline (Me_2NPhNO) oxidoreductase (MNO) (Bystrykh et al., 1993a,b; van Ophem et al., 1993). Nicotinoproteins are enzymes with firmly bound NAD(P), which is not released during catalysis and, therefore, functions as a cofactor rather than as a coenzyme (van Ophem and Duine, 1993). Both nicotinoprotein alcohol dehydrogenases have a broad substrate specificity, but only MNO is able to oxidize methanol. MNO activity, however, is only exhibited when 4-nitrosoaniline derivatives are used as electron acceptors, not with MTT or Cl_2Ind as electron acceptors. Thus,

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Abbreviations. Me_2NPhNO, *N*,*N*′-dimethyl-4-nitrosoaniline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TD-ADH, tetrazolium-dye-linked alcohol dehydrogenase; MeOH-DH, methanol dehydrogenase; MNO, methanol:*N*,*N*′-dimethyl-4-nitrosoaniline oxidoreductase; Cl_2Ind, 2,6-dichloroindophenol.

Enzymes. Methanol:*N*,*N*′-dimethyl-4-nitrosoaniline oxidoreductase (EC 1.1.99.–); alcohol dehydrogenase (acceptor) (EC 1.1.99.8).

what could be the relationship between MNO and the MeOH-DH and TD-ADH activities? Nitrosoanilines are unnatural compounds, which raises questions about the identity of the natural electron acceptor for this type of nicotinoprotein alcohol dehydrogenase. Here we report the resolution of the TD-ADH enzyme complex of *A. methanolica* and some characteristics of the three components.

MATERIALS AND METHODS

Cultivation of bacteria. Bacteria were cultivated on a mineral medium (de Boer et al., 1990) supplemented with 1% (by vol.) methanol for *A. methanolica* NCIMB 11946 and *Mycobacterium gastri* MB19 (Bystrykh et al., 1993b) or 0.5% (by vol.) ethanol for *Rhodococcus erythropolis* DSM 1069 (van Ophem et al., 1991). For the latter two bacteria, yeast extract (0.5%) was added to the medium. Continuous and batch-fed cultivation was carried out at 30°C (or 37°C for *A. methanolica*), pH 7.0, and the oxygen concentration was 80–100% of the air-saturation value. The culture was batch fed with medium containing a threefold higher concentration of methanol and ammonium sulphate, with a dilution rate of 0.1–0.05 h^{-1}. Continuous cultivation was carried out under under methanol at a constant dilution rate (0.05 h^{-1}). Cells were collected by centrifugation, washed with 0.05 M potassium phosphate, pH 6.5, and stored at −20°C.

Crude extract preparation. Frozen cell paste (≈10 g) was suspended in (20 ml) 0.1 M potassium phosphate, pH 6.5 (to prepare extracts showing MeOH-DH activity a buffer of pH 6.3 was required), and the mixture was passed three times through a cooled French pressure cell at 150 MPa. The homogenate was centrifuged for 10 min at 4°C and 25000 *g*, the supernatant being the cell extract.

Enzyme assays. TD-ADH activity was determined at 30°C, as described previously (van Ophem et al., 1991). 900 µl 1 M potassium phosphate, pH 7.5, containing 0.2 mM MTT was mixed with 100 µl sample (0.05–0.5 mg protein). The reduction of MTT was measured at 550 nm. Since endogenous activity was observed, the reaction mixture was allowed to react for 10 min before the substrate (50 mM methanol, unless indicated otherwise) was added. Activities were calculated by substracting the initial rate caused by the endogenous substrate from that caused by the methanol. To assay an individual component for its capacity to reconstitute TD-ADH activity, saturating amounts of the two other components were added. MeOH-DH and MNO activities were determined as described previously with methanol as substrate (Duine et al., 1984; Bystrykh et al., 1993a).

NADH dehydrogenase activity was measured in 1 ml 0.1 M Tris/HCl, pH 8.0, 0.1 mM Cl_2Ind (or 0.2 mM MTT), 0.2 mM NADH, and sample. Cl_2Ind reduction was followed 600 nm and MTT reduction at 550 nm. NADH oxidase activity was measured by adding sample and NADH to 0.05 M Tris/HCl, pH 8.0, and following oxygen consumption and the decrease in absorbance at 340 nm.

Cofactor/coenzyme assays. To establish a role for 5′-deazaflavin as the electron acceptor for TD-ADH or MNO, assays were carried out as indicated above except that MTT was replaced with 5′-deazaflavin (≈7 µM). Activity was determined by following the absorbance at 420 nm (0.3 at the start). Anaerobic measurements were carried out by flushing the cuvette with nitrogen. Experiments with cell free extracts were conducted in 0.1 M potassium phosphate, pH 7.5.

The biological activity of the low-molecular-mass compound (A_{330}) that showed a spectrum similar to those of nicotinamide coenzymes was investigated by determining its capacity to replace NAD in the assay of yeast alcohol dehydrogenase (Boehringer-Mannheim). The assay mixture (1 ml) consisted of 0.2 M Tris/HCl, pH 7.7, 2 mM ethanol or 10 mM formaldehyde, 2 U enzyme, and the A_{330} compound (at a final concentration corresponding to an absorbance of 0.8 at 330 nm). As a control, a mixture lacking the compound but supplemented with 0.2 mM NADH was used. The activity was determined by following the change in absorbance at 330 nm.

Phosphate bond cleavage. Removal of terminal phosphate groups was carried out by incubating the compound in Tris/HCl, pH 7.5, with approximately 10 U alkaline phosphatase (from calf intestine; Boehringer-Mannheim) at 30°C for 30 min. The mixture was kept at 100°C for 3 min and centrifuged to remove aggregated proteins. Cleavage of diphosphate bonds was carried out with phosphodiesterase (from *Crotalus durissus*; Boehringer-Mannheim). Enzyme (1 µl, containing 1 U) was mixed with the compound (100 µl, having an absorbance of ≈0.05 at the absorption maximum) in buffer, pH 7.5, and the mixture was treated as indicated above. The progression of the cleavage was followed by HPLC or TLC (see Isolation of cofactors/coenzymes and chromophores).

Isolation of the TD-ADH components. Ammonium sulphate fractionation was applied to the extract prepared normally from batch-fed-grown *A. methanolica* cells. Since the 30–75% precipitate contained nearly all TD-ADH activity, this precipitate was used as the starting material from which the components were isolated. Proteins were concentrated via centrifugation at 4500 *g*, by means of Filtron Microsep membrane cartridges (cut-off 10 kDa). Protein determinations were carried out with the Bio-Rad protein-assay kit according to the instructions given by the manufacturer. Desalted bovine serum albumin was used as a standard. All three purified components were stored at −80°C. Component 1 was isolated as described previously for MNO (Bystrykh et al., 1993a).

To isolate component 2, the precipitate was dissolved in 0.1 M Tris/H_2SO_4, pH 8.0 (this buffer was used in all subsequent steps). The solution was applied to a gel-filtration column (Superose 12 HR 10/30; Pharmacia) and the fractions that were active when combined with the other two components in the reconstitution assay were collected. Since component 2 did not adhere to Mono S or Mono Q columns, removal of contaminating proteins was carried out by filtrating the collected fractions through a Mono S-Sepharose and a Mono Q-Sepharose HR 5/5 column (Pharmacia).

To isolate component 3, the precipitate was dissolved in 0.1 M potassium phosphate, pH 6.5 (which was the buffer used in all subsequent steps) and the solution applied to a Superose 12 column. Low-molecular-mass-protein fractions that were active when combined with the other two components in the reconstitution assay were filtrated through a Mono S column and the filtrate collected.

Isolation of cofactors/coenzymes and chromophores. The 5′-deazaflavin was isolated according to Daniels et al. (1985). The procedure was routinely applied to *A. methanolica* cells grown batch-fedwise on methanol. Cell paste (5 g) was mixed with 15 ml 1 M NH_4HCO_3/methanol (1:2, by vol.), and the suspension was incubated at 60°C for 30 min, and centrifuged. The supernatant was stored and the extraction repeated twice. The volume of the combined supernatants was reduced to 4 ml by vacuum evaporation. After adding 4 ml ethanol, the mixture was incubated for 30 min on ice, and centrifuged. The supernatant was applied to a Mono Q (5/5) column equilibrated with 0.05 M NH_4HCO_3. Elution occurred with a linear gradient (from 0.05 M to 1 M NH_4HCO_3). Fractions having an absorption maximum at 420 nm were collected and applied to a reverse-phase HPLC column (C_{18} Supelco, 25 mm×10 mm) equilibrated with 0.1 M ammonium acetate, pH 5.5, 3% methanol (solvent A). Elution

occurred at a flow rate of 1 ml/min with isocratic solvent A for 5 min, then with a linear gradient from 100% solvent A to 50% solvent B (80% methanol) over 20 min. The eluate was monitored by fluorescence detection (excitation at 420 nm, emission at 470 nm). As a reference, 5′-deazaflavins with side chains of two or three glutamate residues were used (provided by Dr J. Keltjens, Katholieke Universiteit Nijmegen, The Netherlands). 5-Deazaflavins were detected rapidly and qualitatively by TLC on Kieselgel F60 glass plates, which were developed with methanol/0.1 M ammonium acetate, pH 8.5 (9:1), and the fluorescence was detected after drying the plates. Isolation of the 5′-deazaflavin from components 1 or 3 occurred by heating the solution to 100°C, removing the denatured protein by centrifugation, and applying the supernatant to the reverse-phase HPLC column, after which elution occurred as indicated above.

Compound A_{330} was isolated from purified component 3 or from batch-fed-grown *A. methanolica* cells. The procedure for the latter source was as follows. Cell paste (10 g) was extracted with 40 ml 0.1 M ammonium acetate, pH 5.5/methanol (1:1, by vol.) at 55°C for 15 min. After centrifugation of the mixture, the supernatant was dried in a vacuum evaporator. The solid material was dissolved in a minimal volume of water and an equal volume of ethanol was added. The mixture was incubated at 0°C for 10 min, centrifuged and the supernatant applied to the reverse-phase HPLC column described above. The column was developed with isocratic solvent A for 5 min, a linear gradient from 100% solvent A to 50% solvent C [methanol/acetonitrile (9:1, by vol.)] for 25 min, and a linear gradient from 50% solvent C to 100% solvent C for 5 min. The eluate was monitored by fluorescence detection (excitation at 330 nm, emission at 400 nm). As references, NADH and NADPH (Boehringer-Mannheim) were used. Rapid detection was possible by means of TLC (as indicated above) with methanol/water/acetic acid (9:1:1, by vol.) as developer.

The yellow chromophore from component 2 was extracted in the following way. The protein (5 mg/ml) in 2 ml component 2 solution was precipitated by bringing the solution to 5% trichloroacetic acid. After centrifugation, the pellet was suspended in 10 ml 80% acetone, the suspension incubated for 10 min on ice and centrifuged. The supernatant was concentrated by vacuum evaporation.

Electrophoresis. SDS/PAGE was performed by means of 12% running and 3.5% stacking gel (Laemmli and Favre, 1973). Proteins were denatured in 0.1 M Tris/HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.0002% Bromophenol Blue. The mixture was heated in a boiling water bath for 1 min. To estimate the molecular masses of the proteins, a calibration kit (Combithek; Boehringer-Mannheim) was used. Native electrophoresis was carried out in a similar way, and enzymes were stained by applying assay mixtures to the gels after electrophoresis.

Analytical methods. Absorbance changes (enzyme assays) were measured on a Hitachi 100–60 double-beam spectrophotometer. Fluorescence spectra were determined with a Perkin-Elmer Luminescence spectrometer, type LS-50, and the spectra were converted with FL-data manager. Absorption spectra were measured with an Aminco DW-2000 spectrophotometer. Fluorescence monitoring of the eluate of HPLC columns occurred with a with a Shimadzu RF-551S detector.

RESULTS

Induction and detection of methanol dehydrogenase activities. TD-ADH (40 mU/mg protein) and MNO (5 mU/mg protein) activities could be detected reproducibly in extracts of *A. methanolica* cells obtained from batch-fed cultures grown on methanol. Addition of the respiratory-chain inhibitors KCN (0.5 mM) and sodium azide (1 mM) stimulated TD-ADH activity. MeOH-DH activity (2 mU/mg protein) was detected only when cell disruption occurred in Mes/KOH, pH 6.0–6.3. Addition of NAD led to a twofold increase in activity.

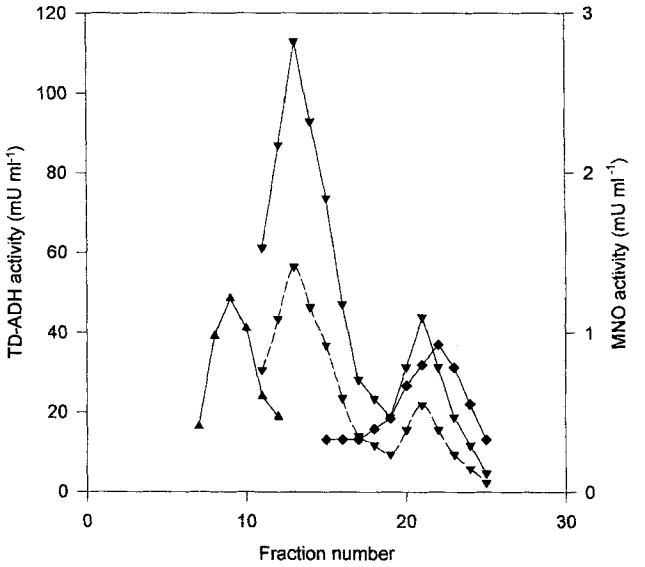

Fig. 1. Gel filtration of the ammonium sulphate precipitate (30–75%) of *A. methanolica* extracts. The precipitate (≈100 mg protein) was dissolved in 0.1 M potassium phosphate, pH 6.5, and applied to a Superose 12 column equilibrated with 0.1 M Tris/HCl, pH 8.0. Fractions (1 ml) were assayed for the presence of one of the three components by testing their ability to reconstitute the combination of the other two components (purified and present in excess) to TD-ADH activity. component 1 (▼), component 2 (▲) and component 3 (◆). The fractions were assayed for MNO activity (▼, broken line).

Table 1. Activities of purified component 1 in different assays. Activities are given relative to the activity obtained with methanol in the MNO assay (corresponding to 5.6 mU/mg protein).

Assay method	Substrate	Electron acceptor	Relative activity
MNO	methanol	Me_2NPhNO	1.0
MNO	formaldehyde	Me_2NPhNO	0.8
TD-ADH	methanol	MTT	0
TD-ADH	formaldehyde	MTT	0
TD-ADH[a]	methanol	MTT	21.6
TD-ADH[a]	formaldehyde	MTT	19.0

[a] Measured in the presence of components 2 and 3.

Resolution of the TD-ADH complex. Ammonium sulphate fractionation of cell-free extracts resulted in recovery of virtually all TD-ADH activity (but not MeOH-DH activity) in the 30–75% saturation fraction. The solubilized pellet could be dialyzed without losing activity but activity was not found in fractions obtained from gel filtration on the Superose 12 column (Fig. 1). However, certain fractions showed TD-ADH activity when they were assayed with a combination of two purified components. With the three combinations, it could be shown that the chromatographic step resolves the complex into three components with different retention times. However, resolution of the complex by this step was not complete since the activity profiles of the components overlapped (Fig. 1), and component 1 activity

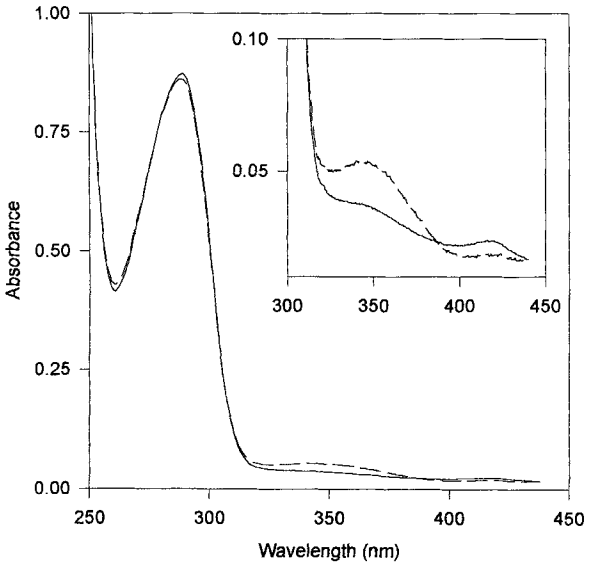

Fig. 2. Absorption spectra of component 1. The spectra were measured in 0.1 M Tris/HCl, pH 7.5, of purified component 1 from chemostat (solid line; 0.4 mg protein/ml) and batch-fed (broken line; 0.3 mg protein/ml) grown cells. The insert shows part of the spectra at expanded scale.

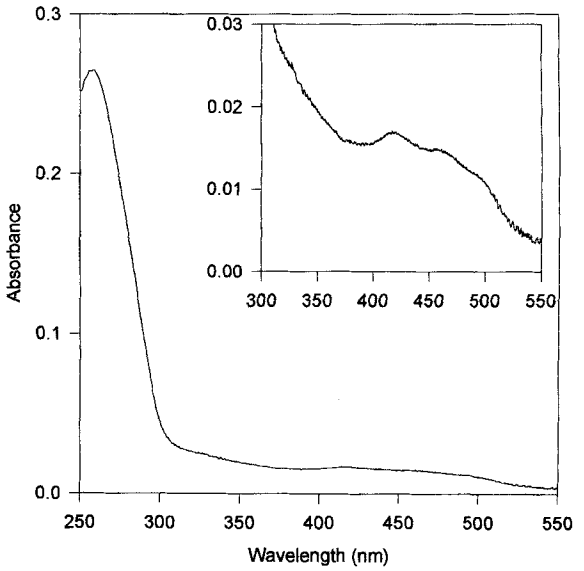

Fig. 3. Absorption spectrum of component 2. The spectrum of purified component 2 (0.05 mg protein/ml) was measured in Tris/HCl, pH 7.5. The insert shows part of the spectrum of purified component 2 at expanded scale.

Table 2. Purification of component 2 from batch-fed-grown *A. methanolica* cells. The activity of component 2 in the fractions was determined by the TD-ADH assay by adding saturating amounts of components 1 and 3 to the sample.

Purification step	Total protein	Specific activity	Recovery	Purification
	mg	mU/mg protein	%	-fold
Crude extract	376	14	100	1
Ammonium sulfate precipitate (30–50%)	323	14	88	1
Superose 12 chromatography	81	46	70	3
Mono S	54	63	64	4
Mono Q	17	121	39	9

Table 3. Purification of component 3 from batch-fed-grown *A. methanolica* cells. The activity of component 3 in the fractions was determined with the TD-ADH assay by adding saturating amounts of components 1 and 2 to the sample.

Purification step	Total protein	Specific activity	Recovery	Purification
	mg	mU/mg protein	%	-fold
Crude extract	306	15	100	1
Ammonium sulfate precipitate (30–75%)	154	9	31	1
Superose 12	0.8	882	16	59
Mono S	0.6	956	7	64

eluted as a major peak (around fraction 13) and as a minor peak (around fraction 21). Purification protocols for each component were devised based on the reconstitution assay, and each component could be purified to a stage at which it was not contaminated by the other two components.

Resolution and reconstitution as described here were not successful with extracts from other bacteria. Extracts from batch-fed-grown *R. erythropolis* and *M. gastri* cells showed TD-ADH activity, as found previously (van Ophem et al., 1991), whereas the resolution step provided fractions containing component 1 (active with components 2 and 3 from *A. methanolica*) but not of fractions showing component 2 or 3 activity. This finding suggests that the nature of TD-ADH in these two bacteria is different from that in *A. methanolica*, or that their components 2 and 3 are more labile after dissociation of the complex. Unusual behaviour of TD-ADH with respect to salt dependency and the amount of extract has been observed for TD-ADH from *M. gastri* but not for that from *R. erythropolis* (van Ophem et al., 1991). These observations suggest that the properties of the TD-ADH complex vary with the organism and perhaps also with the cultivation method applied, and that gram-positive bacteria that produce MNO have TD-ADH activity.

Purification and characterization of the TD-ADH components. *Component 1.* The profile of component 1 for reconstituting TD-ADH activity coincides with that of MNO activity (for the major and minor peaks; Fig. 1). Furthermore, MNO purified according to the previously published procedure (Bystrykh et al., 1993a) could replace component 1 in the reconstitution assay for TD-ADH activity. The substrate specificities of component 1 in the MNO and in the TD-ADH assays were similar, and component 1 did not show TD-ADH activity (Table 1). However, although the behaviour of component 1 was consistent with respect to these properties, it was not with respect to its ultraviolet–visible absorption spectrum (Fig. 2). Whether the difference is related to different growth conditions is unclear. Since the stained SDS/polyacrylamide gels of the preparations were identical, showing one band of 50 kDa, the additional absorption band at 420 nm could originate from a low-molecular-mass compound that remained attached to the MNO in certain cases. On boiling a solution containing such an MNO, in addition to the fluorescence of the detached NADPH, fluorescence was observed with an excitation maximum at 420 nm (data not shown).

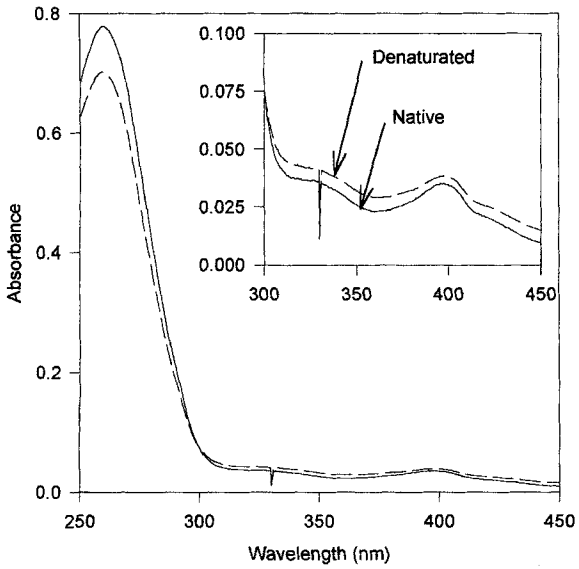

Fig. 4. Absorption spectrum of component 3. The spectrum of purified component 3 (0.01 mg protein/ml), native (——) and heat-denatured (– – –), was measured in 0.1 M Tris/HCl, pH 7.5. The insert shows part of the spectra at expanded scale.

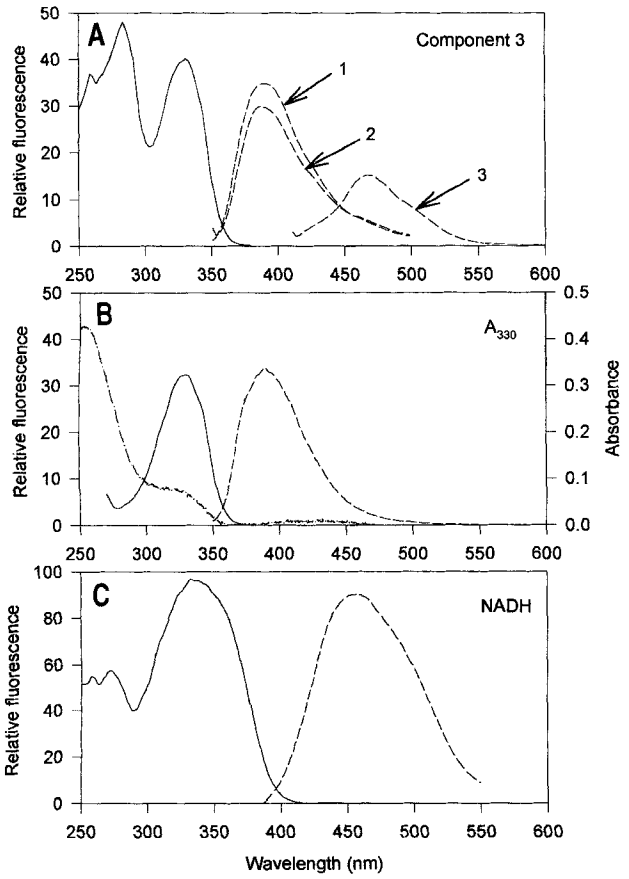

Fig. 5. Fluorescence spectra of component 3, compound A_{330}, and NADH. (A) The excitation spectrum of component 3 (solid line) in 0.1 M Tris/HCl, pH 7.5, was measured at an emission wavelength of 390 nm. The fluorescence emission spectra of component 3 for excitation at 340 (curve 1), 330 (curve 2), and 400 nm (curve 3) are also shown. The excitation (solid line) and emission (broken line) spectra (emission at 390 nm, excitation at 330 nm) of purified compound A_{330} and its absorption spectrum (broken line) are shown in (B), those of NADH (excitation at 340 nm, emission at 460 nm) in (C).

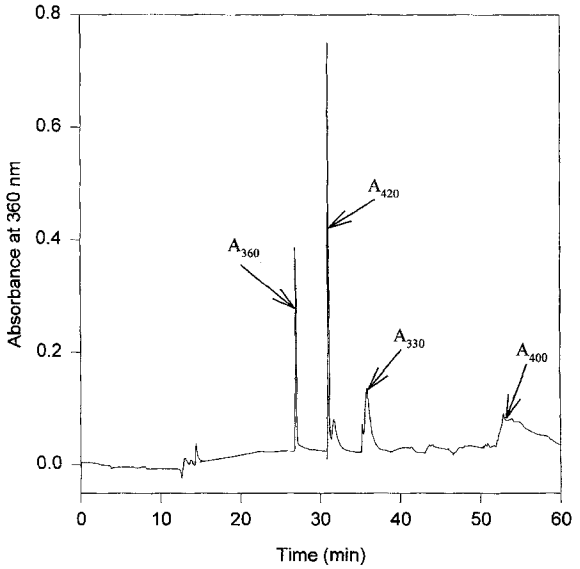

Fig. 6. Reverse-phase HPLC of heat-denatured component 3. Component 3 (0.01 mg/ml) in 0.1 M potassium phosphate, pH 6.5, was denatured by heat and the solution applied to the Supelco C_{18} column. The column was developed as described in Materials and Methods, and the absorbance of the eluate was monitored at 360 nm. The peak fractions were collected and their absorption spectra measured [the eluting compounds are indicated as A_x, where x is the absorption maximum (nm) of the compound].

The substance responsible for the additional fluorescence and absorbance at 420 nm was extracted from the protein and purified. Its chromatographic and spectral properties were identical to those of the 5′-deazaflavin extracted from whole cells and from component 3. Since no differences in activity were observed between preparations of component 1 in which the 5′-deazaflavin was absent or present (in the latter case, based on the molar absorption coefficient of factor F_{420} (Eirich et al., 1978) and the amount of MNO determined with the protein assay, it is estimated that only about 10% of the component 1 molecules contained 5′-deazaflavin), it is concluded that this compound remains sometimes attached to component 1 but that it has no function in the activities measured here for this component.

Component 2. Component 2 was purified as indicated in Table 2. Because it is so large (it elutes in the front fractions of the gel-filtration column, suggesting a molecular mass of >640 kDa), the final preparation is probably not contaminated with other proteins, and therefore the two subunits of 72 kDa and 44 kDa observed with SDS/PAGE probably derive from component 2. Component 2 showed NADH (but not NADPH) dehydrogenase activity with MTT as electron acceptor, and NADH oxidase activty (16% and 4%, respectively, of its activity in the TD-ADH reconstitution assay). The absorption spectrum of component 2 is shown in Fig. 3. Addition of thiol compounds or NAD(P)H did not change it. However, addition of MTT did, suggesting that the chromophore is in a reduced state. Addition of trichloroacetic acid to component 2 and centrifugation gave a pellet and a supernatant. The latter did not contain a chromophore, as judged from the absorption spectrum. Extraction of the pellet with 80% acetone gave a yellow, nonfluorescent solution with an absorption spectrum similar to that of component 2 above 300 nm. Only one yellow spot was seen in the TLC chromatogram, and its retention time did not change when the sample was treated with alkaline phosphatase or phosphodiesterase. Thus, the chromophore of component 2 seems to

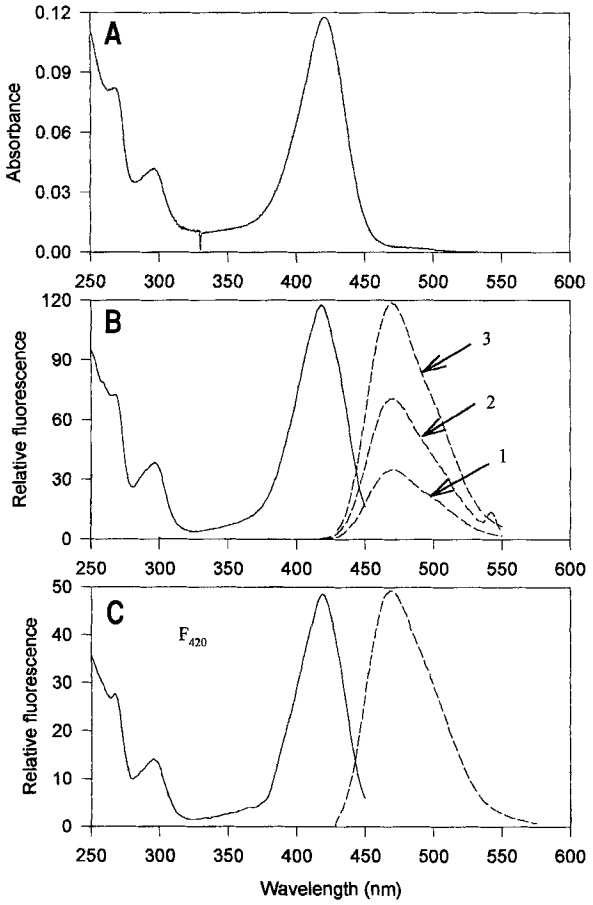

Fig. 7. Absorption and fluorescence spectra of the 5′-deazaflavin purified from *A. methanolica* cells and factor F_{420}. (A) The absorption spectrum of the purified 5′-deazaflavin from batch-fed-grown *A. methanolica* cells was measured in 0.1 M Tris/HCl, pH 7.5. (B) The fluorescence-excitation spectrum (emission at 475 nm; solid line) and the emission spectra (broken lines) with excitation at 270 (curve 1), 300 (curve 2) and 420 nm (curve 3) of purified 5′-deazaflavin as in (A). (C) Fluorescence spectra of factor F_{420} (isolated from *Methanobacterium thermoautotrophicum*) in 0.1 M Tris/HCl, pH 7.5 (excitation spectrum, solid line, with the emission wavelength at 475 nm; emission spectrum, broken line, with the excitation wavelength at 420 nm).

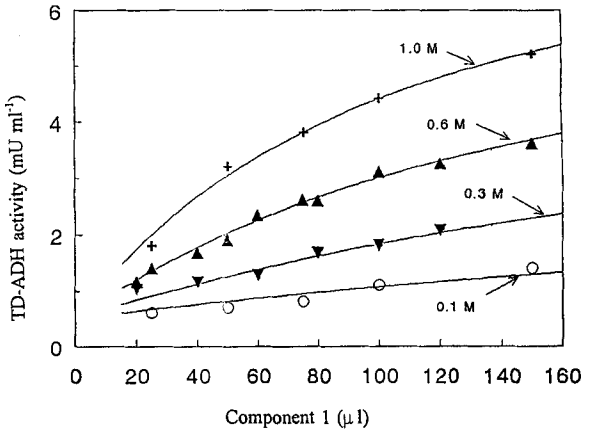

Fig. 8. Reconstitution of TD-ADH activity with varying concentrations of component 1. A mixture of saturating amounts of components 2 and 3 was titrated with varying amounts of component 1 and the initial rate of TD-ADH activity determined with the normal assay. The various concentrations of potassium phosphate at which the experiments were performed are given. The experimental data were fitted with an equation applying to normal Michaelis-Menten-like kinetics.

be an apolar compound in a reduced state, in which phosphate groups are lacking, and which seems to be active as a cofactor in transferring reducing equivalents to MTT.

Component 3. Component 3 was purified as indicated in Table 3. Since it was retained by the membrane when applying the concentration procedure, its molecular mass should be larger than 10 kDa. The preparation appeared to be homogeneous since only one band (whose position corresponds with a molecular mass of 15 kDa) was observed with SDS/PAGE. The absorption spectrum of component 3 showed maxima at 260, 330, 400 nm, and a shoulder at 420 nm (Fig. 4). Two fluorescent chromophores were present in component 3, as judged from the two fluorescence-emission maxima upon excitation at different wavelengths (Fig. 5A). On heat denaturation, a low-molecular-mass substance (which passes through the membrane during the concentration procedure) was liberated, which reduced MTT and Me_2NPhNO. Reverse-phase HPLC of heat-treated component 3 showed the presence of at least four low-molecular-mass compounds (Fig. 6), the compounds designated according to the maximum with the highest wavelength in their absorption spectra (data not shown). Based on its spectral and chromatographic behaviour, compound A_{420} is identical to the 5′-deazaflavin extracted from certain preparations of component 1 and from whole cells (Fig. 7). Since the ratio of the 5′-deazaflavin to the A_{330} compound is low (based on calculations using the molar absorption coefficients of factor F_{420} and NADH), the 5′-deazaflavin in component 3 seems to be a remnant of that present in the original TD-ADH complex. Compound A_{330} showed blue-shifted absorption and fluorescence spectra compared with those of the nicotinamide coenzyme NADH (Fig. 5B and C). Addition of yeast alcohol dehydrogenase and ethanol or formaldehyde, increased or decreased the absorbance, respectively, indicating that compound A_{330} acts as a coenzyme for this enzyme. Since the fluorescence spectra of A_{330} are similar to those of one of the compounds present in component 3 (Fig. 5), and the absorption spectra of native and denatured component 3 are similar (Fig. 4), it seems that compound A_{330} is not a degradation product of NAD(P)H but a natural nicotinamide-like substance. This was confirmed by the observation that treatment with phosphodiesterase abolished its activity with yeast alcohol dehydrogenase and changed its retention time on the HPLC column (data not shown). Compounds A_{400} and A_{360} were not further investigated because they did not exert any effect in the assays of the *A. methanolica* enzymes.

Reconstitution of TD-ADH. On mixing the three components, TD-ADH activity appeared, but not that of MeOH-DH. On titrating a mixture of two components with the third one, Michaelis-Menten saturation behaviour was observed (Fig. 8). Since high phosphate and sulphate concentrations stimulate TD-ADH activity (van Ophem et al., 1991), the effects of these conditions were studied on the reconstitution process. On increasing the phosphate concentration from 0.1 M to 1.0 M, the V_{max} increased twofold whereas the K_m for component 2 and methanol remained unchanged, that for component 1 decreased fourfold, and that for component 3 decreased twofold (data not shown). It seems, therefore, that the salting-out effect generally ascribed to phosphate and sulphate improves the interaction between components 1 and 3, leading to a better performance of the TD-ADH complex.

Characteristics of the 5′-deazaflavin. The 5′-deazaflavin extracted from *A. methanolica*, *M. gastri* and *R. erythropolis* cells appeared to have pH-dependent absorption and fluorescence

spectra identical to those of the 5′-deazaflavin isolated from methanogens, factor F_{420} (Fig. 7), indicating that the chromophores are identical. However, since the retention times on the reverse-phase HPLC column were different, the polyglutamate side chains seem to be not identical. The 5′-deazaflavin was not homogeneous, showing three or four peaks in the chromatogram, corresponding with species having a side chain of 4−7 glutamyl residues, that with five residues being the main component. Identical patterns were observed for the 5′-deazaflavin extracted from whole cells and that from the corresponding TD-ADH components, but the pattern varied between organisms (unpublished results).

No 5′-deazaflavin was found in the culture fluid or in the cell debris, half of the amount occurring in the 75% ammonium sulphate precipitate of the *A. methanolica* extract, the other half remaining in the supernatant. Upon gel filtration of the solubilized precipitate, the distribution of the 5′-deazaflavin was as follows. The majority of the 5′-deazaflavin eluted with component 3 and about 3% with component 1. The 5′-deazaflavin could not replace MTT as the electron acceptor in the TD-ADH assay, under aerobic or anaerobic conditions. However, it became reduced when crude extracts of *A. methanolica* plus methanol were added (2 mU/mg protein). The activity was abolished when ammonium sulphate fractionation was applied, and no reconstitution was achieved by recombining fractions.

DISCUSSION

The results indicate that TD-ADH is a multienzyme complex which can be dissociated into three protein components by applying normal chromatographic purification steps. Each component could be purified to a stage where it was no longer contaminated with the other two components, as judged from SDS/PAGE patterns and the necessity to add the other two components to obtain TD-ADH activity. The Michaelis-Menten behaviour observed in kinetic studies on TD-ADH, in which the concentrations of the components were changed, indicate that complex formation between the three components has to take place for TD-ADH activity to be generated. The stimulation of association of the components by adding salts could indicate that the TD-ADH complex is situated in the cell at a location with low water activity.

The role of each component in TD-ADH activity could be as follows. Based on the arguments mentioned above, the alcohol oxidation step in TD-ADH is carried out by component 1 (MNO). Since component 2 displays low NADH dehydrogenase (diaphorase) activity with MTT, it is tempting to speculate that it functions as an electron acceptor for component 1, with component 3 acting as a mediator in this. How and whether the redox compounds (the 5′-deazaflavin, the nicotinamide-like compound, and the yellow chromophore) act as cofactors in the complex remain to be elucidated. The coupling of the TD-ADH complex to the respiratory chain also awaits investigation. This coupling may involve additional components since combination of the three components yielded TD-ADH, but not the apparently more delicate MeOH-DH complex. Perhaps the latter contains another redox-active component that is able to transfer electrons to Cl_2Ind *in vitro* and to the respiratory chain *in vivo*. Despite the lack of knowledge on these aspects, the present results allow a conclusion to be made on the significance of TD-ADH. No alcohol dehydrogenases can be detected in *A. methanolica* during growth on primary aliphatic alcohols other than the two nicotinoprotein alcohol dehydrogenases (van Ophem et al., 1993). Mutants of this organism in which active component 1 (MNO) or component 2 were lacking did not grow on methanol (and lower aliphatic alcohols), and revertants regained the activities of the components (Hektor and Dijkhuizen, 1996). It is concluded, therefore, that the MNO-containing TD-ADH complex is the sole catalyst for methanol oxidation in *A. methanolica* and probably for lower-aliphatic-alcohol oxidation in other gram-positive bacteria in which MNO or the complex have been detected (van Ophem et al., 1991; Nagy et al., 1995).

5′-Deazaflavins are not only present in methanogens (known as factor F_{420}) but have also been found in some gram-positive bacteria, e.g. *Mycobacterium* (Naraoka et al., 1984), *Streptomyces* and *Nocardia* (Daniels et al., 1985). In addition to a role in methanogenesis (reviewed by DiMarco et al., 1990), 5′-deazaflavin seems to be involved in photoreactivation of ultraviolet-irradiated DNA (Minato and Werbin, 1972; Eker et al., 1981), and in chlorotetracycline biosynthesis (McCormic and Morton, 1982). It was reported that 5′-deazaflavin can act as a coenzyme for hitherto unknown NAD(P)-independent alcohol dehydrogenases of methanogens (Widdel and Wolfe, 1989; Kunow et al., 1994) and glucose-6-phosphate dehydrogenase of *Mycobacterium smegmatis* (Purwantini and Daniels, 1996). Because of the low redox potential of 5′-deazaflavins and the high reactivity of reduced deazaflavins with flavins, a role of them as free coenzymes is difficult to envisage on thermodynamical grounds, especially in aerobic bacteria (however, there may be no problem as a cofactor since the protein to which it binds may increase the redox potential and prevent reaction with flavins). Here it is shown that 5′-deazaflavin was present in the TD-ADH complex of *A. methanolica* and in other bacteria containing such a complex. Although some reduction took place of 5′-deazaflavin in crude extracts of *A. methanolica* when methanol was added, the disappearance of this activity upon fractionation of the extract and the inability of TD-ADH to donate reducing equivalents to the compound could indicate that the 5′-deazaflavin has another role. In this context, 5′-deazaflavin-dependent glucose-6-phosphate dehydrogenase activity was observed in *A. methanolica* (Misset-Smits, M. and Duine, J. A., unpublished results). Thus, it would be interesting to investigate whether the 5′-deazaflavin-containing component 3, being essential for activity of the TD-ADH complex in alcohol oxidation *in vitro*, has a role in mediating electron transfer from the glucose-6-phosphate dehydrogenase.

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