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

Leonid V. Bystrykh1, 2, Natalya I. Govorukhina1, 2, Lubbert Dijkhuizen2 and Johannis A. Duine1

1 Department of Microbiology and Enzymology, Delft University of Technology, Delft, The Netherlands
2 Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Haren, The Netherlands

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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 pyrroloquinolino-quinone-containing alcohol dehydrogenase. This complexity is illustrated by the diverse methanol-oxidizing activities described for the actinomycete *Amoyleolatopsis methanola*. Kato et al. (1975) reported that extracts of methanol-grown cells exhibit phenazine methosulphate/2,6-dichloroindolophenol (ClInd) methanol dehydrogenase activity. Subsequently, Duine et al. (1984) reported the presence of a methanol dehydrogenase (MeOH-DH) in a multienzyme complex, displaying a ClInd-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 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 ClInd as electron acceptors. Thus,
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 sulfate. The culture was batch fed with medium containing a threefold higher concentration of methanol and ammonium sulfate. The culture was batch fed with medium containing a threefold higher concentration of methanol and ammonium sulfate. The culture was batch fed with medium containing a threefold higher concentration of methanol and ammonium sulfate.

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, and the supernatant was stored at -20°C.

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 subtracting 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 other two components were added. MeOH-DH and MNO activities were determined as described previously with methanol as substrate (Duijne 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 Cl̄Ind (or 0.2 mM MTT), 0.2 mM NADH, and sample. Cl̄Ind 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 (A330) 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 (Boehminger-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 A330 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 Filter 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/HCl, 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 filtering 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 NH₄HCO₃/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 NH₄HCO₃. Elution occurred with a linear gradient (from 0.05 M to 1 M NH₄HCO₃). Fractions having an absorption maximum at 420 nm were collected and applied to a reverse-phase HPLC column (C₄, 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 isotropic 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 solvent B (80% methanol) over 20 min. The eluate was monitored 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 A30 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 isotropic 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 470 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 (Coomassie Blue, 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 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.

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 these 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

---

**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 3.6 mU/mg protein).

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Substrate</th>
<th>Electron acceptor</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNO</td>
<td>methanol</td>
<td>Me$_2$NH$_2$NO</td>
<td>1.0</td>
</tr>
<tr>
<td>MNO</td>
<td>formaldehyde</td>
<td>Me$_2$NH$_2$NO</td>
<td>0.8</td>
</tr>
<tr>
<td>TD-ADH</td>
<td>methanol</td>
<td>MTT</td>
<td>0</td>
</tr>
<tr>
<td>TD-ADH</td>
<td>formaldehyde</td>
<td>MTT</td>
<td>0</td>
</tr>
<tr>
<td>TD-ADH*</td>
<td>methanol</td>
<td>MTT</td>
<td>21.6</td>
</tr>
<tr>
<td>TD-ADH*</td>
<td>formaldehyde</td>
<td>MTT</td>
<td>19.0</td>
</tr>
</tbody>
</table>

*a* Measured in the presence of components 2 and 3.
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.

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.

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

cultivated 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.

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.

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

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 A1200 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 A1200 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 C18 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 Ax, 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 F420, 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 activity (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, non-fluorescent 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
phores were present in component 3, as judged from the two fluorescence-emission maxima upon excitation at different wavelengths (Fig. 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 13 kDa) was observed with SDS/PAGE. The absorption spectrum of component 3 showed maxima at 260, 330, 400 nm, with the emission wavelength at 420 nm (Fig. 4). Two fluorescent chromophores 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 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 MT and MeNPhNO. 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 absorption spectra (data not shown). Based on its spectral and chromatographic behaviour, compound A, 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, compound is low (based on calculations using the molar absorption coefficients of factor F and NADH), the 5'-deazaflavin in component 3 seems to be a remnant of that present in the original TD-ADH complex. Compound A, 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, acts as a coenzyme for this enzyme. Since the fluorescence spectra of A, 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, 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 and A, 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 Vmax increased twofold whereas the Km 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

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**Fig. 7. Absorption and fluorescence spectra of the 5'-deazaflavin purified from A. methanolica cells and factor F**

(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 (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).

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**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.
spectra identical to those of the 5'-deazaflavin isolated from methanogens, factor F_m (Fig. 7), indicating that the chromophores are identical. However, since the retention times on the reverse-phase HPLC column were different, the polyglutamate phores are identical. However, since the retention times on the remaining in the supernatant. Upon gel filtration of the solubi-

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 (unpub-

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 solubi-

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 compo-

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 appar-

No 5'-deazaflavin were different, the polyglutamate phores 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 (unpub-

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