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The formaldehyde dehydrogenase of *Rhodococcus erythropolis*, a trimeric enzyme requiring a cofactor and active with alcohols

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During growth on compounds containing methyl groups a formaldehyde dehydrogenase is induced in the gram-positive bacteria *Rhodococcus erythropolis*.

1. This formaldehyde dehydrogenase has been purified to homogeneity using affinity chromatography and permeation chromatography. The isoelectric point of the enzyme was 4.7. The molar mass of the native enzyme was determined as 130000 g/mol. Sodium dodecyl sulfate gel electrophoresis yielded a single subunit with a molar mass of 44000 g/mol. These results, together with cross-linking experiments which yielded monomer, dimer, and trimer bands, are consistent with a trimeric subunit structure of the formaldehyde dehydrogenase.

2. A heat-stable cofactor of low molar mass was required for activity with formaldehyde as substrate. This cofactor was found to be oxidizable, but active only in its reduced form. Preparative electrofocusing revealed that the cofactor is a weak acid with a pK of about 6.5.

3. The enzyme was active with the homologous series of the primary alcohols, ethanol up to octanol, without requiring the presence of the cofactor. A mutant without formaldehyde dehydrogenase activity was not impaired in its growth with ethanol as substrate.

It is suggested that the alcohols mimic the true substrate of the formaldehyde dehydrogenase, which could be a hydroxymethyl derivative of the cofactor, resulting from the addition of formaldehyde.

Formaldehyde is known to be oxidized to formate either as free formaldehyde or as an enlarged molecule [1, 2]. In the latter case formaldehyde reacts non-enzymatically with glutathione or tetrahydrofolate and the resulting adducts are the true substrates for the formaldehyde dehydrogenases. Besides glutathione and tetrahydrofolate a third but as yet not characterized cofactor has been shown to occur in *Methylococcus capsulatus* during growth on methane [3].

We found that in *Rhodococcus erythropolis* a formaldehyde dehydrogenase activity is present, which is linked to the metabolism of compounds containing methyl groups, e.g. methoxybenzoic acids or methylamines [4]. Preliminary studies in crude extracts revealed that this activity is lost on dialysis and that glutathione and tetrahydrofolate failed to reactivate the enzyme activity. Instead, boiled crude extract of *R. erythropolis* could restore the formaldehyde dehydrogenase activity. This apparent cofactor requirement attracted our interest and prompted us to study the formaldehyde dehydrogenase system in more detail. In this paper we describe purification and characteristics of the formaldehyde dehydrogenase, which is composed of three subunits. In addition we describe features of the cofactor required for activity of the enzyme with formaldehyde, but not for activity with alcohols.

MATERIALS AND METHODS

Strain, mutant isolation and cultivation

Rhodococcus erythropolis (DSM 1069) was obtained from the Deutsche Stammsammlung für Mikroorganismen (Göttingen). Mutants were raised with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described [5]. They were selected for their inability to utilize methylamine as a nitrogen source. The rationale for this procedure was that non-growth with methylamine would be due to suicide of the culture resulting from the accumulated formaldehyde. The carbon and energy source was 5 g/l glucose. Those clones unable to grow with 0.5 g/l methylamine, but not affected in their growth with ammonium chloride, were screened for loss of formaldehyde dehydrogenase activity. The formaldehyde dehydrogenase was induced by transferring pregrown cells (1% glucose, 48 h) to fresh medium containing 2 mM 3,4-dimethoxybenzoic acid (16 h).

Growth medium and culture conditions were as described [4]. For large-scale cultivation on 3,4-dimethoxybenzoic acid (2 mM) a fermenter with 8 l working volume (Braun, Biostat V) was used. After consumption of the substrate two further additions of 3,4-dimethoxybenzoic acid, 2 mM each time, were made to give a final yield of 12 g wet weight of cells.

Enzyme assay

If not otherwise stated, formaldehyde dehydrogenase activity was assayed at 30 °C in 250 mM sodium phosphate pH 8, with 1 mM NAD, 3.8 mM dithiothreitol, 6 mM formaldehyde and 30 µl cofactor preparation (in a final volume of 1 ml). The reactions were started by the addition of formaldehyde and the initial NADH formation was recorded. The alcohol dehydrogenase assay was identical with the formaldehyde dehydrogenase assay, except that formaldehyde was replaced by 0.5 M ethanol and the cofactor preparation was omitted.

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Protein was determined after precipitation according to Bensadoun and Weinstein [6]. The specific activity is expressed in nmol min^{-1} mg^{-1}.

Preparation of cofactor

Cell-free extract (about 15 mg protein/ml) was boiled for 15 min and clarified by centrifugation (20 min, $10000 \times g$). 2.5 ml resulting supernatant was applied to a prepacked Sephadex G-25 column (Pharmacia, PD-10). The column was eluted with water and the fractions eluting between 6 ml and 9 ml were collected as the cofactor preparation.

Purification of formaldehyde dehydrogenase

The buffer used throughout the purification procedure was 0.1 M sodium phosphate pH 7.5 containing 0.01% sodium azide. All operations were done at 4°C, flow rates were 5–6 ml/h. To disrupt the cells, a washed suspension was frozen in an X-Press (AB-Biox, Nacka, Sweden) to −18°C and passed through an orifice of 0.8 mm diameter. After thawing, the resulting homogenate was centrifuged for 30 min at $10000 \times g$ and the supernatant used as cell-free extract. Of this, 15 ml were dialyzed for 8 h and applied to a 5′-AMP-Sepharose column (7×2.6 cm). The column was washed, followed by elution of bound material with a linear gradient of 0 to 4 mM NAD (240 ml). The active fractions were combined, concentrated by ultrafiltration (YM10, Amicon) and applied to a Sephadex G-200 column (93×1.6 cm). The collected active fractions were again concentrated by use of a YM10 membrane and used as purified enzyme.

Determination of the molar mass

This was determined by calibration of the Sephadex G-200 column (see purification) with ribonuclease A, chymotrypsinogen A, ovalbumin, albumin, aldolase, catalase, ferritin and thyroglobulin. K_{av} values were determined according to [7].

Cross-linking

A modified method of Davies and Stark [8] was employed for cross-linking with dimethyladipimidate and dimethylsuberimidate. The imidate solutions (4 mg/ml 0.04 M triethanolamine pH 9) were prepared just prior to use to reduce hydrolysis [9], and 12 µl of this solution mixed with 20 µl (25 µg) formaldehyde dehydrogenase. The linker disuccinimidylsuberate was dissolved in dimethylsulfoxide (3.4 mg/ml). 2 µl of this solution was added to 20 µl formaldehyde dehydrogenase solution. Controls consisted of formaldehyde dehydrogenase plus triethanolamine buffer or dimethylsulfoxide respectively. The assays were incubated for 30 min at 30°C to allow cross-linking. Then 60 µl 10 mM 2-amino-2-hydroxymethyl-1,3-propanediol pH 8, 1 mM (ethylenedinitrilo)tetraacetic acid sodium salt, 0.7 M 2-mercaptoethanol, 1% sodium dodecyl sulfate were added, and assays incubated for 1 h at 37°C to prepare them for sodium dodecyl sulfate gel electrophoresis.

Electrophoretic separations

Electrophoresis under non-denaturing conditions was done in a discontinuous polyacrylamide gel. The stacking gel was 4% polyacrylamide, 0.062 M Tris (2-amino-2-hydroxymethylpropane-1,3-diol), 0.062 M HCl, pH 6.7, the separating gel 7.3% polyacrylamide, 0.37 M Tris, 0.06 M HCl, pH 8.9. The electrophoresis buffer was 0.05 M Tris, 0.38 M glycine, pH 8.3.

For electrophoresis in the presence of sodium dodecyl sulfate a modified system of Laemmli [10] was used. The stacking gel was 4% polyacrylamide, 0.1% sodium dodecyl sulfate, 0.12 M Tris, pH 6.8, the separating gel 8% polyacrylamide, 0.1% sodium dodecyl sulfate, 0.375 M Tris, pH 8.8. The electrophoresis buffer was that used for electrophoresis under non-denaturing conditions, but additionally contained 0.1% sodium dodecyl sulfate.

To increase the sample density and to observe the migration, a drop of 0.5 mg bromphenol blue/ml glycerol was added to samples prior to application to the slab gels. After electrophoresis the gels were stained in 0.05% Coomassie blue R-250, 25% propan-2-ol, 10% acetic acid and destained in 10% acetic acid.

Isoelectric focusing

Analytical electrofocusing was done in 240-µm-thick polyacrylamide gels. The gels consisted of 4% acrylamide (total), 5% bisacrylamide and contained 6 M urea and 3% ampholines pH 2–10 (LKB, Bromma, Sweden). Standards (with pI) were amyloglucosidase (3.5), ferritin (4.4), bovine albumin (4.7), β-lactoglobulin (5.3), conalbumin (5.9), horse myoglobulin (7.3), whale myoglobulin (8.3) and ribonuclease (9.4).

For preparative electrofocusing 4.5 ml cofactor preparation were mixed with the stabilizing gel slurry (5% Sephadex G-75, superfine) together with 3% ampholines pH 2–10. The final bed volume was about 70 ml. After focusing overnight 30 fractions were obtained by use of a grid. Their pH was determined and they were eluted with 3 ml 0.1 M sodium phosphate pH 8. They were assayed for cofactor by using 400 µl elutate, 4 µg formaldehyde dehydrogenase, 250 µmol sodium phosphate pH 8, 1 µmol NAD and 6 µmol formaldehyde (total volume 1 ml).

Formaldehyde, acetaldehyde and formate determination

For formaldehyde determination a modification of Nash's method was used [11]. The reagent solution consisted of 15 g ammonium acetate, 0.3 ml acetic acid and 0.2 ml acetyl acetone/100 ml water. Samples were mixed with an equal volume of reagent solution, incubated for 5 min at 60°C and the resulting absorption at 412 nm determined. The formaldehyde standard was prepared by boiling paraformaldehyde overnight in a closed flask. Acetaldehyde was determined by gas chromatography.

Formate was determined with formate dehydrogenase of yeast. The assay system contained per milliliter: 50 µmol sodium phosphate pH 6.5, 7 µmol NAD (lithium salt), 1.2 U formate dehydrogenase (Boehringer, Mannheim, FRG) and 200 µl sample.

RESULTS

Enzyme activities in a formaldehyde-accumulating mutant

To clarify the apparent linkage between formaldehyde oxidation and ethanol oxidation in cells metabolizing methyl groups [4], a mutant without formaldehyde dehydrogenase activity was isolated by the suicide selection procedure. This mutant grew normally with ethanol as the substrate (final dry

Table 1. *Purification of the formaldehyde dehydrogenase of* R. erythropolis *with copurification of alcohol dehydrogenase activity*

Step	Formaldehyde dehydrogenase						Alcohol dehydrogenase			
	total volume	total protein	total activity	specific activity	yield	purification factor	total activity	specific activity	yield	purification factor
	cm^3	mg	μmol/min	U/mg	%		μmol/min	U/mg	%	
Extraction	24	182	15.3	0.09	100	1	4.2	0.023	100	1
5'AMP-Sepharose	0.86	2.4	8.3	3.46	54.1	38.4	2.2	0.92	52.3	40.0
Gel permeation	0.90	1.1	4.6	4.40	30.1	48.9	1.3	1.22	30.9	52.2

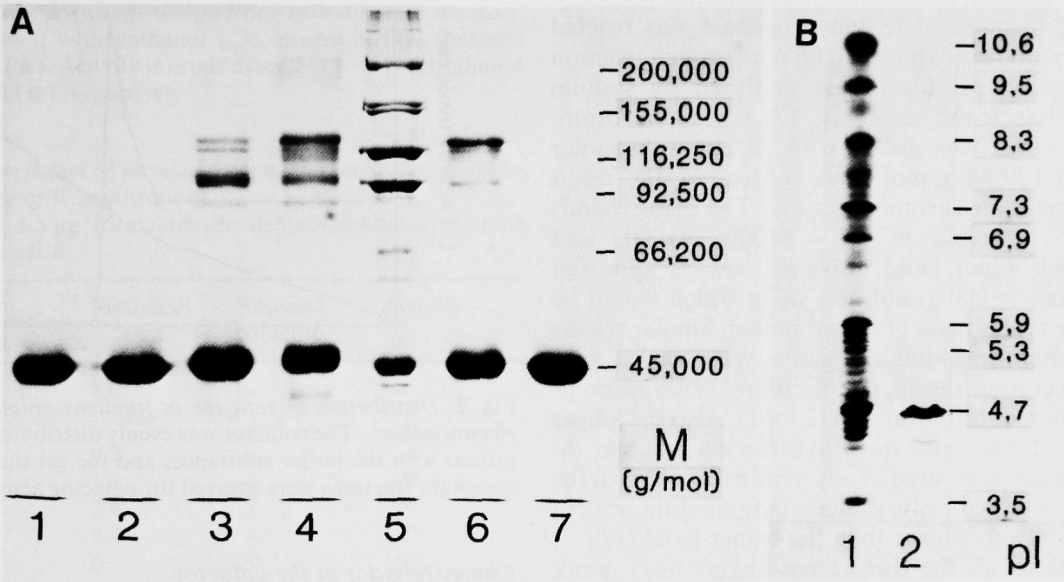

Fig. 1. *(A) Sodium dodecyl sulfate gel electrophoresis of the formaldehyde dehydrogenase of* R. erythropolis *and cross-linked species of the enzyme. (B) Isoelectric focusing of purified formaldehyde dehydrogenase.* (A) Each assay consisted of 25 μg enzyme. Lane 1, without addition; lane 2, (control) unreacted in imidate reaction buffer; lane 3, reacted with 45 μg dimethyladipimidate; lane 4, reacted with 45 μg dimethylsuberimidate; lane 5, molar mass markers; lane 6, reacted with 8 μg succinimidylsuberate; lane 7, (control) unreacted in succinimidylsuberate reaction buffer. (B) Lane 1, standards; lane 2, enzyme

weight: 56 mg/0.39 g ethanol) and 3,4-dihydroxybenzoic acid, which is the degradation product of 3,4-dimethoxybenzoic acid [5]. Upon inoculation of this mutant on 3,4-dimethoxybenzoic acid no substantial growth was observed, but 50 μmol substrate were oxidized within 2 days, accompanied by the accumulation of 30 μmol formaldehyde. A crude extract of this formaldehyde-accumulating mutant contained neither formaldehyde dehydrogenase activity nor an activity with ethanol as substrate. This is an indication that in cells metabolizing methyl groups a formaldehyde dehydrogenase is present which is also active with ethanol.

Purification of the formaldehyde dehydrogenase

Different chromatographic procedures have been attempted, including ion-exchange chromatography and hydrophobic chromatography. Finally, an affinity step has been chosen, which, in combination with the permeation chromatography, permitted a rapid and simple purification procedure (Table 1). It characteristically yielded about 1 mg enzyme from 200 mg protein applied as crude extract. A small fraction (about 5%) of the activity was never retained on 5'-AMP-Sepharose. This was not due to an overloading of the column, and it is not clear whether this fraction contained partially denatured enzyme.

During the purification procedure the dehydrogenase activity with ethanol as the substrate was also followed. As outlined in Table 1, yields and purification factors for both activities were almost the same. In ion-exchange experiments alcohol dehydrogenase activity also copurified with formaldehyde dehydrogenase activity. This indicates again that both activities reside in one protein.

Homogeneity of the protein

The final enzyme preparation was run on polyacrylamide gels. This resulted in broad banding with the occasional formation of two bands. Furthermore, the migration velocity was dependent on the amount of enzyme applied. These anomalies could be partially eliminated by the addition of dithiothreitol to samples prior to application, suggesting oxidation of the enzyme during the run. In accordance with this assumption the migration of the enzyme was not distorted when denaturing conditions were used. In 8% polyacrylamide (Fig. 1A), as well as in 12% polyacrylamide, one single band was formed, thus establishing the homogeneity of the final preparation. Finally the protein was electrofocused using a pH range 3.5–9.5. Again only one band was obtained (Fig. 1B) corresponding to an isoelectric point of the formaldehyde dehydrogenase of 4.7.

Molar mass and subunit structure

The apparent molar mass of the native enzyme was determined by permeation chromatography using Sephadex G-200. By reference to the standard curve, a distribution factor according to a molar mass of 120000 – 140000 g/mol was obtained. The subunit mass, as determined by sodium dodecyl sulfate gel electrophoresis, yielded one substructure of 44000 – 47000 g/mol. Both these mass determinations are in accordance with a trimeric structure of the formaldehyde dehydrogenase.

Since odd-numbered enzyme structures are rare [12], cross-linking experiments with homo-bifunctional reagents were done to provide further evidence of this unusual property. For this purpose formaldehyde dehydrogenase was reacted with dimethyladipimidate (which is 0.86 nm between reaction centers) and reaction products were analyzed by sodium dodecyl sulfate gel electrophoresis (Fig. 1 A, lane 3). No prominent reaction product was visible with an apparent molar mass larger than 130000 g/mol, thus confirming the result obtained by permeation chromatography. The major bands corresponded to proteins of M = 44000, 95000, and 130000 g/mol. The dimer band, however, always appeared with M larger than 86000 g/mol, the value which would be expected from the molar mass of the monomer. Similar results were obtained when cross-linking assays were varied with respect to protein concentration, use of different enzyme preparations or inclusion of dithiothreitol (2 mM). Also the longer cross-linkers ($\approx$ 1.1 nm span) dimethylsuberimidate and disuccinimidylsuberate were used. They yielded band patterns resembling that obtained with dimethyladipimidate, except the dimer band was less intense than the trimer band (Fig. 1, lanes 4 and 6). With all the three cross-linkers used, weak bands were also visible with apparent molar masses smaller than the main species. They might be due to interchain rearrangement reactions, charge alterations or presence of weak protease activity.

Products and stoichiometry of the formaldehyde dehydrogenase reaction

In a 1-ml standard assay formaldehyde was reacted with formaldehyde dehydrogenase for 16 min until the formation of 120 nmol NADH. To determine the expected formate, an assay with formate dehydrogenase of yeast was used. However, no formate could be detected when aliquots of the formaldehyde dehydrogenase assay were directly used for formate determination. This was not due to interference of the formate assay with components of the formaldehyde dehydrogenase assay, since an internal standard of 100 nmol sodium formate included in the formaldehyde dehydrogenase assay could be quantitatively recovered when samples were taken at different times during the formaldehyde dehydrogenase reaction. When, however, aliquots of the formaldehyde dehydrogenase assay were pretreated by boiling for 5 min, 98% of the expected formate was detectable. This suggests that the product of the formaldehyde reaction is an unstable form of formate, which is not identical with free formate.

The consumed formaldehyde was determined in a separate experiment using a colorimetric assay. It yielded a consumption of 480 nmol formaldehyde when 460 nmol NADH were formed (as calculated with $\varepsilon_{365} = 3409\ M^{-1}\ cm^{-1}$). These results suggest that the oxidation of formaldehyde gave rise to an equimolar amount of the non-enzymatically detectable form of formate accompanied by the reduction of NAD.

Table 2. *Effect of dithiothreitol on the activity of the formaldehyde dehydrogenase system*

Formaldehyde dehydrogenase	Cofactor	Dithiothreitol	Relative activity
µg	µl	mM	%
2	20	3.8	100
2	20	–	0
2	–	3.8	8
2	–	–	0
–	20	3.8	0

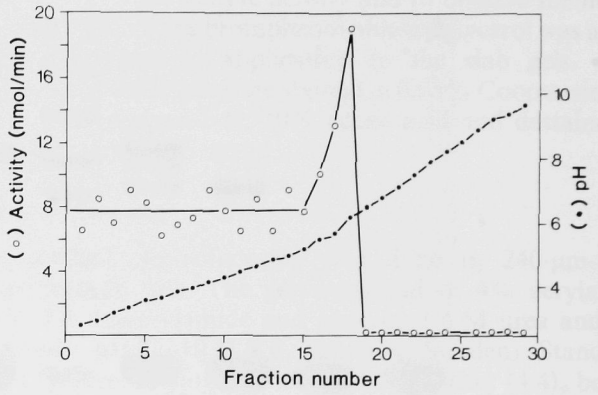

Fig. 2. *Distribution of cofactor in fractions obtained by preparative electrofocusing*. The cofactor was evenly distributed by mixing preparations with the buffer substances and the gel slurry. After focusing overnight fractions were assayed for cofactor activity

Characteristics of the cofactor

Cofactor preparations lost their activity within three days. In subsequent experiments we found that storage under nitrogen or addition of mercaptoethanol or dithiothreitol sustained the activity for longer periods. Furthermore it was possible to reactivate inactive cofactor preparations by reduction. Therefore, for routine analysis dithiothreitol (3.8 mM) was included in formaldehyde dehydrogenase assays. Concentrations higher than 7 mM were found to be inhibitory, most probably due to inactivation of the enzyme. The effect of dithiothreitol on the formaldehyde dehydrogenase system is shown in Table 2.

To further characterize the cofactor with respect to its electric properties, preparative electrofocusing was performed. As evident from the migration behaviour (Fig. 2), the cofactor was not protonable but negatively charged in the alkaline region at pH higher than 6. Thus the cofactor is not amphoteric but behaves like a weak acid with pK around 6.5. Since the cofactor passed ultrafiltration membranes of 0.12 nm pore size, its molar mass is smaller than 1000 g/mol.

Activity with alcohols and kinetics

In an assay where formaldehyde and cofactor were replaced by 340 mM ethanol, addition of the isolated formaldehyde dehydrogenase resulted in NADH formation. After formation of NADH, assays contained acetaldehyde as identified by gas chromatography. It was formed in equimolar amounts (0.20 µmol/ml acetaldehyde corresponding to 0.23 µmol/ml NADH). Thus ethanol served as substrate without any cofactor being present (consequently dithiothreitol was also not necessary in assays). Besides ethanol the homologous series of the primary alcohols propan-1-ol up to

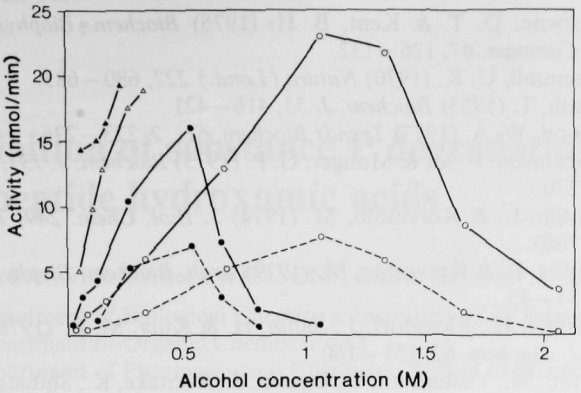

Fig. 3. *Formaldehyde dehydrogenase activity with different alcohols.* Pure enzyme (——), with enthanol (○), propanol (●), butanol (△), and pentanol (▲). Activity in crude extract (– – – –) with ethanol (○), and propanol (●) respectively

Table 3. *Effect of methanol on formaldehyde oxidation by the formaldehyde dehydrogenase of* R. erythropolis
Assays contained 1.5 μg formaldehyde dehydrogenase in 50 mM sodium phosphate pH 8

Formaldehyde	Methanol	Reduced cofactor	Activity
mM	M	μl	%
6	–	–	0
6	0.2	–	4
6	1.2	–	9
6	2.4	–	16
–	2.4	–	0
6	0	20	100

pentan-1-ol were also utilized. Hexan-1-ol and octan-1-ol were utilized as well, though detailed measurements with these longer chain alcohols were impossible owing to their solubility barrier.

The purified enzyme was not saturable with ethanol within a concentration of 1 M (Fig. 3). In this respect the formaldehyde dehydrogenase of *R. erythropolis* resembles the alcohol dehydrogenase of yeast, which also requires very high ethanol concentrations for activity [13]. The apparent rapid dissociation of the enzyme-product complex was not due to an artefact of the formaldehyde dehydrogenase preparation, since very similar results were also found in crude extracts (Fig. 3).

Influence of methanol on formaldehyde oxidation

Though ethanol and the higher alcohols were oxidized without the cofactor being present, methanol was not used as a substrate by the formaldehyde dehydrogenase (Table 3). However, when methanol was included in an assay instead of the reduced cofactor, formaldehyde was oxidized at a significant rate as followed by the NADH production. It therefore follows that methanol exerts an influence on formaldehyde oxidation by the enzyme, which might by similar to the influence of the cofactor.

DISCUSSION

We previously reported on the physiological significance of the formaldehyde dehydrogenase activity in *R. erythropolis* [4]. Thus, the enzyme is present only when methylamines or methoxylated benzoic acids are metabolized by the organism, thereby generating formaldehyde. The suicide mutant described in this paper is further proof of the necessity of this enzyme when formaldehyde is generated. The biochemical investigations described reveal some notable characteristics of the formaldehyde dehydrogenase. This concerns the fact that (a) the enzyme is a trimer, (b) it requires a reduced cofactor for activity and (c) is also active toward alcohols.

The trimeric nature of several enzymes is well established (reviewed in [12]), and no hints of an inferior catalytic activity or stability have been found. The formaldehyde dehydrogenases of liver [14], pea seeds [15], yeast [16], and several bacteria [3, 17] are investigated with respect to their subunit composition. All seem to be composed of two subunits. The evidence obtained for the *R. erythropolis* enzyme points to a trimer, but no conclusions about the arrangement of the subunits can be drawn. Possibly the molar mass of the dimer, which appears always too high, has to do with the subunit arrangement of the enzyme. In this respect it is further remarkable that upon use of the longer cross-linkers the trimer band was more intense than the dimer band, though reaction conditions and chemistry of dimethyladipimidate and dimethylsuberimidate are the same.

The chemical nature of the cofactor necessary for formaldehyde oxidation is still unsolved. Owing to its reducibility it would be natural to suggest a sulfhydryl group as the reactive group. However, an attempt to obtain chemical proof for sulfhydryl groups failed (no data shown) as did assays to show formation of a thiol ester bond. In accordance with this, the pK value is too low as compared to the naturally occurring thiols glutathione, coenzyme A or lipoic acid [21].

With respect to the role of the cofactor during formaldehyde oxidation the activity of the formaldehyde dehydrogenase with alcohols could be instructive. This concerns especially the fact that, in contrast to formaldehyde oxidation, the alcohol oxidation is independent of the presence of the cofactor. This suggests that the alcohols exhibit features of the natural substrate which could be a hydroxymethyl derivative of formaldehyde plus cofactor. Two further hints for such an adduct are the partial replacement of the cofactor by methanol, which reacts with formaldehyde to the semiacetal CH_3-O-CH_2OH, and the fact that the product of formaldehyde oxidation is not the free formate. In this respect the formaldehyde dehydrogenase system of *R. erythropolis* shares similarities to the glutathione-dependent formaldehyde dehydrogenases. Thus, Uotila and Koivusalo [14] demonstrated that glutathione reacts non-enzymatically with formaldehyde to a hydroxymethyl derivative, giving the substrate for the liver enzyme. Furthermore, the product of this oxidation is a formyl adduct, which is readily hydrolyzable as is the oxidation product of the *R. erythropolis* system. However, definite conclusions will only be possible after purification and characterization of the cofactor. Recently a formaldehyde dehydrogenase of *Pseudomonas putida* has been described [20], which also reacts with alcohols. However, the situation with this enzyme is different, since no cofactor seems to be required for formaldehyde oxidation and methanol or ethanol does not serve as a substrate, though longer alcohols do.

The cofactor for activity of the *R. erythropolis* enzyme might add a new cofactor to those already known to be involved in formaldehyde oxidation. A variety of systems are realized in bacteria (reviewed in [1]) including those rare cases where free formaldehyde serves as a substrate [17, 20]. The investigated formaldehyde dehydrogenases of eucaryotes re-

quire glutathione for activity. It therefore appears that systems which use the free formaldehyde are less common than those where formaldehyde is handled as an 'enlarged molecule'.

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