Purification and Characterization of NADP⁺-Dependent 5,10-Methylenetetrahydrofolate Dehydrogenase from *Peptostreptococcus productus* Marburg

GERT WOHLFARTH, GABRIELE GEERLIGS, AND GABRIELE DIEKERT*

Institut für Mikrobiologie, Universität Stuttgart, Azenbergstrasse 18, D-7000 Stuttgart 1, Federal Republic of Germany

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The 5,10-methylenetetrahydrofolate dehydrogenase of heterotrophically grown *Peptostreptococcus productus* Marburg was purified to apparent homogeneity. The purified enzyme catalyzed the reversible oxidation of methylenetetrahydrofolate with NADP⁺ as the electron acceptor at a specific activity of 627 U/mg of protein. The $K_m$ values for methylenetetrahydrofolate and for NADP⁺ were 27 and 113 μM, respectively. The enzyme, which lacked 5,10-methylenetetrahydrofolate cyclohydrolase activity, was insensitive to oxygen and was thermolabile at temperatures above 40°C. The apparent molecular mass of the enzyme was estimated by gel filtration to be 66 kDa. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a single subunit of 34 kDa, accounting for a dimeric $a_2$ structure of the enzyme. Kinetic studies on the initial reaction velocities with different concentrations of both substrates in the absence and presence of NADPH as the reaction product were interpreted to indicate that the enzyme followed a sequential reaction mechanism. After gentle ultracentrifugation of crude extracts, the enzyme was recovered to >95% in the soluble (supernatant) fraction. Sodium (10 μM to 10 mM) had no effect on enzymatic activity. The data were taken to indicate that the enzyme was similar to the methylenetetrahydrofolate dehydrogenases of other homoacetogenic bacteria and that the enzyme is not involved in energy conservation of *P. productus*.

Homoacetogenic bacteria are strictly anaerobic eubacteria that mediate the total synthesis of acetate from CO₂ in their energy metabolism. The methyl group of acetate is formed from CO₂ via formate and tetrahydrofolate-bound C-1 intermediates; synthesis of the carboxyl group proceeds via a bound carbon monoxide (for recent reviews on homoacetogens and their energy metabolism, see references 3, 7, 13, and 20). Most homoacetogenic bacteria are able to grow on H₂ plus CO₂ as energy sources. Therefore, reduction of CO₂ to acetate must be coupled with a net formation of ATP. Studies with *Acetobacterium woodii* (4) led to the assumption that ATP synthesis is coupled to the formation of the methyl group of acetate via a chemiosmotic mechanism. For the homoacetogen *Peptostreptococcus productus* (9) and other homoacetogens (10, 21), evidence was presented that an electrochemical sodium gradient formed upon synthesis of the methyl group from formate could play an essential role in energy conservation. The sodium-dependent reaction in methyl group synthesis is not yet known. Therefore, we studied the enzymes mediating methyl formation. Of particular interest are the enzymes that catalyze reactions sufficiently exergonic to be possibly coupled to a sodium translocation across the cytoplasmic membrane. These reactions are, at least under standard conditions and with CO or H₂ as an electron donor, the reduction of methylenetetrahydrofolate to methyltetrahydrofolate and the formation of methylenetetrahydrofolate from methylenetetrahydrofolate. The former reaction is the most exergonic step in methyl group synthesis. Studies of the methylenetetrahydrofolate reductase in vesicles of *Clostridium therm autotrophicum* (11) and of the same enzyme purified from *P. productus* (19) indicated that the enzyme could be associated with the membrane, thus pointing to its proposed role in energy conservation. However, the methylenetetrahydrofolate reduction was not dependent on sodium (19).

Here we report on the isolation and properties of the methylenetetrahydrofolate dehydrogenase to elucidate the possible role of this enzyme in energy conservation of homoacetogenic bacteria. Our data can be interpreted as excluding such a role in the homoacetogen *P. productus*. This conclusion is supported by an earlier observation, that in *A. woodii* the formation of the methyl group from formaldehyde, which does not involve methylenetetrahydrofolate dehydrogenase, was also sodium dependent (10).

**MATERIALS AND METHODS**

**Materials.** Tetrahydrofolic acid, DNase II, and Dowex 50 WX 8, 100/200 mesh, were from Serva (Heidelberg, Federal Republic of Germany). Formaldehyde, 2-mercaptoethanol, and folic acid were from Fluka (Neu-Ulm, Federal Republic of Germany). NAD(P)⁺ and flavin adenine dinucleotide were from Boehringer (Mannheim, Federal Republic of Germany). The chromatography and column materials except re- active yellow 26-agarose were purchased from Pharmacia (Uppsala, Sweden). Centricon 30 tubes were from Amicon (Danvers, Mass.). Reactive yellow 26-agarose and all other chemicals of the highest available purity were obtained from Sigma (Deisenhofen, Federal Republic of Germany).

**Analytical methods.** Methylenetetrahydrofolate dehydrogenase was assayed routinely by photometric determination of the reduction of NADP⁺ and formation of methylenetetrahydrofolate from methylenetetrahydrofolate (19). Methyltetrahydrofolate reductase was measured by monitoring the formation of NADPH at 350 nm (ε = 5.6 mM⁻¹ cm⁻¹ for NADPH and 24.9 mM⁻¹ cm⁻¹ for methylenetetrahydrofolate) at 37°C. The assays were conducted aerobically in cuvettes filled with 1 ml of anaerobi cally prepared 100 mM potassium phosphate buffer (pH 5.5).
or 100 mM Tris-HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 0.5 mM tetrahydrofolic acid, 10 mM formaldehyde, and 0.3 mM NADP⁺. Methylene tetrahydrofolate was formed nonenzymatically from tetrahydrofolate and formaldehyde (18). Only 25% of the tetrahydrofolate initially added were converted to biologically active methylene tetrahydrofolate, since (i) two stereoisomers of tetrahydrofolate are present in the commercially available substance and (ii) part of the tetrahydrofolate was in its oxidized state as dihydrofolate (19). The concentration of convertible methylene tetrahydrofolate was determined as described earlier (19) by limiting the tetrahydrofolate concentration in the methylene tetrahydrofolate reductase and dehydrogenase assays with excesses of the other substrates (NADH and NADP⁺) and formaldehyde. The reaction was started by the addition of protein. The kinetic properties of the enzyme were analyzed by varying the concentrations of methylene tetrahydrofolate and NADP⁺ as the substrates and NADPH as the product of the reaction (see Fig. 3 and 4). The methylene tetrahydrofolate concentrations of convertible substrate are given in Results and Discussion.

To exclude the presence of methylene tetrahydrofolate cyclohydrolylase activity in the purified enzyme, the cyclohydrolylase reaction was measured by the addition of crude extracts to methylene tetrahydrofolate dehydrogenase assays, which had been previously incubated with purified dehydrogenase until the methylene tetrahydrofolate had been completely converted to methenyl tetrahydrofolate, i.e., until a constant absorption was reached. The decrease of the absorption due to the conversion of methylene tetrahydrofolate to formyl tetrahydrofolate indicated the presence of cyclohydrolylase in the crude extracts (0.38 μmol/min/mg of protein at pH 7.5).

Protein was determined with bovine serum albumin as the standard by the Coomassie blue method (Bio-Rad, Munich, Federal Republic of Germany). Iron was determined in a spectrophotometric assay as described earlier (6).

The molecular weight of the native enzyme was determined by gel filtration on a Superdex 75 column (1.6 by 60 cm). The sample and elution buffer was 20 mM Tris-HCl (pH 7.5) containing 0.5 mM dithiothreitol and 0.15 M KCl. The standard proteins were RNase A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (66.0 kDa), alkaline phosphatase (80.0 kDa), and β-galactosidase (85.0 kDa).

The molecular weight of the subunit and the subunit composition of the enzyme were determined with a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel stained with Coomassie blue. The standard proteins were α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), egg albumin (45.0 kDa), and bovine serum albumin (66.0 kDa).

To investigate the effect of sodium on methylene tetrahydrofolate dehydrogenase activity, the assay buffer was replaced by 0.1 M ammonium acetate (pH 5.5). The assay mixture was passed through a Dowex 50 WX 8 column (0.5 by 5 cm) preequilibrated with 0.1 M ammonium acetate. With this procedure, the sodium content was decreased to a concentration of <10 μM. The concentration of sodium was estimated by heating a drop of liquid on a platinum inoculation loop in the blue flame of a Bunsen burner. A change of the flame's color to yellow indicated the presence of sodium. NaCl solutions were used as the standards. No color change of the flame was observed when the cuvette content was treated in that way. The detection limit of the method was near 5 μM sodium. The reversibility of the methylene tetrahydrofolate dehydrogenase reaction was studied in the same assay system in the presence and absence of added sodium (5 mM). The following substrate concentrations were used: methylene tetrahydrofolate (‘biologically active’), 50 μM; and NADP⁺, 100 μM. After the equilibrium of the reaction was reached, 100 μM NADPH was added. The subsequent decrease in absorption indicated the reoxidation of NADPH with methylene tetrahydrofolate as the electron acceptor. No reverse reaction was observed when the enzyme was irreversibly inactivated by heating the cuvette to 60°C for 10 min prior to the addition of NADPH.

**Purification of the methylene tetrahydrofolate dehydrogenase.** *P. productus* (ATCC 43917) cells were grown on glucose medium as described earlier (8). The cells were harvested by centrifugation in the late logarithmic growth phase and stored frozen at −20°C.

(i) **Step 1. Preparation of crude extracts and ammonium sulfate treatment.** Frozen cells were suspended in an equal volume of 50 mM Tris-HCl (pH 7.5) containing 5 mM mercaptoethanol, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mg of DNase II per ml. The suspension was passed twice through a French pressure cell at 100,000 kPa. The crude extract was centrifuged at 80,000 × g and 4°C for 60 min. The supernatant was stirred on ice, and ice-cold 3.2 M ammonium sulfate (pH 7.0) was added to a final concentration of 1.9 M. After being stirred on ice for 10 min, the mixture was centrifuged at 20,000 × g for 10 min to remove precipitated protein.

(ii) **Step 2. Chromatography on phenyl-Sepharose.** The supernatant was passed through a phenyl-Sepharose Fast Flow column (1.0 by 15 cm) preequilibrated with 1.6 M ammonium sulfate in basic buffer (20 mM Tris-HCl [pH 7.5] containing 0.5 mM dithiothreitol). The column was washed with 1.6 and 1.1 M ammonium sulfate (60 ml each) in basic buffer. The dehydrogenase was eluted with 0.8 M ammonium sulfate in basic buffer.

(iii) **Step 3. Chromatography on reactive yellow 86-agarose.** Fractions containing dehydrogenase were pooled, desalted, and concentrated by centrifugation with a Centricon 30. The enzyme solution was applied to a reactive yellow 86-agarose column (1.0 by 6.0 cm) preequilibrated with basic buffer. The column was washed with ~20 ml of basic buffer, and the dehydrogenase was then eluted with 3 M folic acid in basic buffer.

(iv) **Step 4. Chromatography on Mono Q.** Fractions containing dehydrogenase were pooled, and 9 volumes of modified basic buffer (20 mM Tris-HCl [pH 8.5] containing 0.5 mM dithiothreitol) were added. The solution was applied to a Mono Q column (1.0 by 10 cm) preequilibrated with modified basic buffer. The dehydrogenase was eluted with a linear gradient from 0 to 0.5 M KCl in modified basic buffer at KCl concentration of approximately 0.3 M. Fractions containing dehydrogenase were concentrated with a Centricon 30 and stored frozen at −20°C.

**RESULTS AND DISCUSSION**

**Purification of 5,10-methylene tetrahydrofolate dehydrogenase.** The enzyme was purified from *P. productus* to a specific activity of 627 U/mg (pH 5.5). The purification procedure is summarized in Table 1. The enzyme was stable against oxygen, at least in the presence of dithiothreitol or mercaptoethanol; therefore, the purification could be performed in the presence of oxygen. Anaerobic purification increased neither the specific activity nor the purification.
yield. The addition of phenylmethylsulfonyl fluoride to the cell extracts prevented the degradation of enzyme activity due to proteases present in the extracts. After treatment of the crude extracts with ammonium sulfate (see step 1 of the purification procedure), no more protease activity occurred.

The purified methylenetetrahydrofolate dehydrogenase mediated the oxidation of methylenetetrahydrofolate to methenyltetrahydrofolate with NADP$^+$ as the electron acceptor; no activity was observed with NAD$^+$, flavin adenine dinucleotide, or ferredoxin from Clostridium pasteurianum as cosubstrate. The reaction was reversible, as indicated by the oxidation of NADPH with methylenetetrahydrofolate formed by the enzyme in the reaction assay under appropriate conditions (Fig. 1). From the equilibrium concentrations of the reactants in the experiment shown in Fig. 1, the standard redox potential of the couple methenyl-/methylene-tetrahydrofolate was estimated to be near $-300$ mV, confirming the value that has been reported before for this couple (7). The enzyme was judged to be homogeneous on the basis of polyacrylamide gel electrophoresis in the absence and presence of SDS (Fig. 2). No cyclohydrodrolase activity was detected in the purified enzyme, in contrast to the enzyme of the homoacetogen Clostridium thermoaceticum (12, 15). Under the conditions routinely used, the enzyme oxidized methylenetetrahydrofolate to methenyltetrahydrofolate; a subsequent conversion of methenyltetrahydrofolate to formyltetrahydrofolate was observed only when crude extracts of the bacterium were added to the assays. The specific activity of the cyclohydrodrolase in the extracts was near 0.38 U/mg at pH 7.5.

**Molecular composition.** Gel filtration of the purified enzyme in comparison with molecular size standards indicated an apparent molecular size of the enzyme of $\approx 64$ kDa (data not shown). SDS-polyacrylamide gel electrophoresis revealed the presence of a single subunit with an apparent molecular size of $34$ kDa (Fig. 2). Therefore, a dimeric $\alpha_2$ structure can be envisaged. A similar structure was observed for the enzymes purified from other homoacetogens, such as A. woodii (55 kDa; subunits, $\approx 26.5$ kDa), C. thermoaceticum (15) (55 kDa; subunits, $\approx 30.0$ kDa), and C. formicoaceticum (14) (60 kDa; subunits, $\approx 32$ kDa).

**Involvement in energy conservation.** The sodium-dependent methyl group formation from formate (9) and the indications for an ATP synthesis coupled to methyl group formation (4) led to the assumption that one (or more) of the enzymes involved in methyl group formation is responsible for the coupling mechanism, probably via the formation of an electrochemical sodium gradient. Since the reaction methylenetetrahydrofolate$^+$ + $H_2$ $\rightarrow$ methenyltetrahydrofolate + $H^+$ is sufficiently exergonic under standard conditions ($\Delta G^\circ = -23$ kJ/mol) (7) to account for the electrogenic translocation of one monovalent cation across the cytoplasmic membrane, the methylenetetrahydrofolate reduction could possibly be a sodium-dependent, energy-yielding reaction in methyl group formation. Our data, however, provide the following evidence against this hypothesis.

(i) The electron donor for the methylenetetrahydrofolate

**FIG. 1.** Reversibility of the methylenetetrahydrofolate dehydrogenase reaction. The reaction mixture (pH 5.5) contained initially 50 $\mu$M methylenetetrahydrofolate and 100 $\mu$M NADP$^+$. The reaction was started by the addition of 120 $\mu$g of purified dehydrogenase and was recorded spectrophotometrically at 350 nm. At the time indicated by the arrow, 100 $\mu$M NADPH was added. For further details, see Materials and Methods.

**FIG. 2.** SDS-polyacrylamide gel electrophoresis of the purified methylenetetrahydrofolate dehydrogenase from P. productus. Electrophoresis was carried out in the presence of 12% polyacrylamide with $\approx 10$ $\mu$g of protein. The gel was stained with Coomassie blue G-250 and scanned with a flat-bed scanner. Molecular weight markers were $\alpha$-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa).
reduction is most probably NADPH \( (E^\text{m} = -320 \text{ mV}) \) rather than ferredoxin \( (E^\text{m} = -420 \text{ mV}) \), as indicated by the specificity of the backward reaction for NADP\(^+\) as the electron acceptor. With NADPH as the electron donor, however, the \( \Delta G^\circ \) value of the above reaction is \(-4.9 \text{ kJ/mol}\), which is not sufficient (at least under standard conditions) to account for the translocation of a monovalent cation.

(ii) When cell extracts obtained after gentle lysis of the cells with lysozyme (Cellosyl; Hoechst AG, Frankfurt, Federal Republic of Germany) were centrifuged for 120 min at 80,000 \( \times \) g, the supernatant (i.e., the soluble fraction) contained more than 95% of the total methylenetetrahydrofolate dehydrogenase activity; less than 5% was recovered in the pellet. The methylenetetrahydrofolate dehydrogenase, which was used as a control, was found to about 40% in the particulate (pellet) fraction (19). This result was interpreted to indicate that the methylenetetrahydrofolate dehydrogenase is not associated with the membrane, as would be required for the involvement in energy conservation via a chemiosmotic mechanism.

(iii) The activity of the purified dehydrogenase in both reaction directions was not decreased in the absence of added sodium, i.e., if the sodium concentration was less than 10 \( \mu M \). This is an argument against the assumption that this reaction could be coupled to a sodium translocation.

(iv) Earlier investigations with \( A. \) woodii (10) indicated that the formation of the methyl group from formaldehyde, which is nonenzymatically converted to methylenetetrahydrofolate, is also sodium dependent. Although this could not be confirmed for \( P. \) productus (data not shown), this finding argues against a role of the methylenetetrahydrofolate dehydrogenase in energy conservation.

For the reasons described above, involvement of this enzyme in the energy conservation of homoacetogenic bacteria is very unlikely. The energy-yielding reaction is probably either the reduction of methylenetetrahydrofolate to methylenetetrahydrofolate (19) or a later step in the synthesis of the methyl group.

Catalytic properties. The enzyme did not exhibit a distinct pH optimum; in a pH range of 5.5 to 8.5, the activity was almost the same, with a slight increase toward higher pH values. Since at pH >8 the methylenetetrahydrofolate was unstable (see also reference 17), the activity could not be measured accurately beyond this limit. The enzyme appeared to be heat sensitive at temperatures of \( >40^\circ \text{C} \). Incubation at \( 50^\circ \text{C} \) for 10 min led to a 90% loss of the activity; at \( 60^\circ \text{C} \), a complete loss of activity was observed. A low thermal stability was also reported for the methylenetetrahydrofolate dehydrogenase of \( C. \) formicoaceticum (14), a heat-stable methylenetetrahydrofolate dehydrogenase was purified whereas from the thermophilic homoacetogen \( C. \) thermoaceticum (15).

The initial reaction velocities of methylenetetrahydrofolate oxidation with NADP\(^+\) as the electron acceptor were studied in the presence of different methylenetetrahydrofolate and NADP\(^+\) concentrations. When the approximate \( V_{\text{max}} \) values obtained from the Lineweaver-Burk plots (in the absence of NADPH) were replotted against the concentrations of both substrates, the \( K_m \) values were determined to be 27 \( \mu M \) for methylenetetrahydrofolate and 113 \( \mu M \) for NADP\(^+\). The \( V_{\text{max}} \) value was approximately 150% of the velocity obtained under the conditions routinely used for the determination of enzyme activity.

In the Lineweaver-Burk plots, the lines reflecting the dependence of \( 1/v \) versus \( 1/[S] \) were parallel with different given concentrations of the second substrate, indicating a ping-pong catalytic mechanism of the enzyme (Fig. 3). This was earlier observed for both reaction directions of the methylenetetrahydrofolate dehydrogenase purified from \( A. \) woodii (17). However, kinetic studies like those depicted in Fig. 3 or described in reference 17 are not sufficient to allow unambiguous determination of the reaction mechanism. Therefore, we investigated the initial reaction velocities in the presence of different NADPH concentrations. Assuming a ping-pong reaction mechanism, NADPH should be an inhibitor competitive to methylenetetrahydrofolate, since both electron donors would compete for the same redox center in the enzyme (16). The studies showed, however, that the inhibition pattern was of the mixed type (Fig. 4B) rather than competitive. This was also true with respect to NADP\(^+\) (Fig. 4A). The data therefore argue against a
ping-pong reaction mechanism. The patterns observed indicate a sequential catalytic mechanism, with the intermediary formation of a compulsory-order ternary complex (16).

A ping-pong reaction mechanism would imply the presence of a reducible prosthetic group in the enzyme. Therefore, we performed spectroscopic studies with the enzyme to confirm or exclude the presence of such a cofactor, which could be either an enzyme-bound pyridine nucleotide, a paraquinolinoquinone, a pterin (flavin or molybdopterin), a metal, or a still unknown prosthetic group. Iron could be excluded, since the iron content was determined to be ≤0.25 mol/mol of subunit. Other metals could be present; however, the finding that complexing agents and cyanide did not affect the enzyme activity argues against a metal. If a metal is the cofactor, it appears to be not easily removable from the enzyme. The absorption spectrum of the purified enzyme (Fig. 5) exhibited properties typical for proteins without any detectable prosthetic group, with an absorption maximum at ~280 nm. The addition of sodium borohydride did not influence the spectrum, thus excluding the presence of an enzyme-bound pyridine nucleotide. Paraquinolinoquinone would have been easily detectable in the spectrum and can therefore be excluded. The enzyme did not show any UV light-induced fluorescence, as is known for molybdopterin enzymes; the fact that azide did not inhibit the enzyme supports the assumption that molybdenum is not the cofactor. When the enzyme was heated to 100°C for 10 min in the absence and presence of trichloroacetic acid or HCl (10 mM each), no distinct absorption spectrum was observed in the supernatant after centrifugation. The data are taken to indicate the absence of any cofactor in the enzyme.

Since the spectroscopic investigations led to negative results with respect to the presence of a prosthetic group, inhibition studies with a variety of potential inhibitors were performed to obtain indirect information on the absence or presence of such a reducible cofactor (Table 2). Complexing agents such as EDTA, o-phenanthroline, and neocuproine in concentrations of 10 mM each did not affect enzyme activity. Zinc has been reported to inhibit the reaction catalyzed by the purified enzyme from A. woodii (17). Inhibition was also observed with the P. productus enzyme, the extent of inhibition being similar to that of the A. woodii enzyme. Of the other divalent cations tested (Cu2+, Co2+, Ba2+, Mn2+, Mg2+, Cd2+, Fe2+, and Ni2+), only nickel had a slightly inhibitory effect (~50% inhibition at a concentration of 20 mM). Reagents for the detection of reactive SH groups such as p-chloromercuriphenylsulfonic acid and sodium arsenite (see also reference 18) had no inhibitory effect; only quick-silver ions (HgCl2) were inhibitory (Table 2). The latter

![Figure 4](image_url)

**FIG. 4.** Lineweaver-Burk kinetic plots of 1/[S] versus 1/v of methylenetetrahydrofolate oxidation with NADP+ catalyzed by methylenetetrahydrofolate dehydrogenase (60 ng/ml of assay mixture; 627 U/mg) in the presence of different concentrations of NADPH. (A) Substrate, NADP+; methylenetetrahydrofolate concentration, 63 μM. (B) Substrate, methylenetetrahydrofolate; NADP+ concentration, 100 μM. Assay conditions: temperature, 37°C; pH 5.5; methylenetetrahydrofolate, NADP+, and NADPH concentrations as indicated. v is given as micromoles of substrate converted per minute in the assay.

![Figure 5](image_url)

**FIG. 5.** Absorption spectrum of the purified methylenetetrahydrofolate dehydrogenase (1.3 mg/ml) in 20 mM Tris-HCl buffer (pH 8.5) containing 0.5 mM dithiothreitol.
TABLE 2. Effects of inhibitors on activity of purified methylenetetrahydrofolate dehydrogenase

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* Percentage of the control value in the absence of inhibitor (100% = 627 U/mg).

inhibition could be due to the same effect that zinc or nickel ions had on the enzyme activity rather than to the binding of Hg2+ to SH groups. No effect on enzyme activity was observed with hydroquinone, azide, cyanide, or N-ethylmaleimide at 10 mM each. Clearly inhibitory were reagents for carbonyl functions. Hydroxylamine and phenylhydrazine inhibited the enzyme ~50% at concentrations of about 5 to 10 mM. The data were interpreted to indicate the absence of a prosthetic group in the enzyme. The effect of zinc, nickel, and quicksilver could be the complexing of side chains of amino acids involved in the catalytic mechanism. The inhibition by hydroxylamine and phenylhydrazine could be due to a reaction with carbonyl groups; this conclusion, however, is speculative and remains to be proven.

The kinetic studies at first glance indicate a ping-pong reaction mechanism, although further kinetic studies support the idea of a sequential mechanism. In addition, we failed in our efforts to demonstrate the presence of a reducible cofactor, which would be a prerequisite for a ping-pong mechanism. For these reasons, a sequential mechanism is proposed for the enzyme from P. productus. Earlier studies on the methylenetetrahydrofolate dehydrogenase of the homocetogen A. woodii were interpreted to indicate a ping-pong reaction mechanism (17). However, extended kinetic studies on the reaction mechanism of this enzyme are lacking. Other examples of enzymes "pretending" a ping-pong mechanism, such as hexokinase (2), amino acid oxidase (5), and 6-phosphofructokinase (5), have been reported. A sequential mechanism was also reported recently for the methylenetetrahydrofolate dehydrogenase from yeast cells (1).

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