Characterization of Methylamine Dehydrogenase from Bacterium W3A1. Interaction with Reductants and Amino-Containing Compounds†

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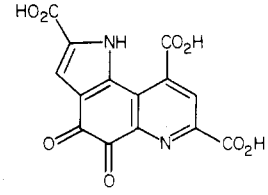
ABSTRACT: Methylamine dehydrogenase from bacterium W3A1 is composed of two subunits of unequal molecular weight. From sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a molecular weight of 45 000 is obtained for the larger subunit and 15 500 for the smaller one. Gel chromatography of the native enzyme yields a molecular weight of 127 000. These data suggest that the enzyme has a tetrameric structure of type A_2B_2. The enzyme contains a chromophore, with absorbance maximum at 429 nm, which is covalently linked to the lower molecular weight subunit. Anaerobic titration of the enzyme with dithionite or methylamine results initially in an increase in absorbance at 429 nm which continues until 1 molar equiv of reductant is added. The enzyme at this stage has an electron spin resonance spectrum of 7.5 G width and a g value of 2.0086. Subsequent addition of reducing agent leads to a decrease in absorbance at 429 nm and a concomitant increase in absorbance at 330 nm. This process also consumes 1 mol of reductant/mol of enzyme. The results are consistent with the titration of 2 mol of cofactor/mol of enzyme. Although the intermediate radical species regains its original oxidized spectrum in the presence of oxygen, the fully reduced enzyme is inert in this regard. The cofactor–peptide can be separated from the larger subunit by DEAE-cellulose chromatography in the presence of urea. This smaller peptide exhibits an absorbance maximum at 424 nm and is reduced by dithionite in a 1:1 molar ratio. Admission of oxygen leads to reoxidation of the chromophore. When reduced, the cofactor is fluorescent with excitation and emission maxima at 320 and 410 nm, respectively. Addition of ammonium ion to solutions of the enzyme produces a color change from a greenish to a reddish hue, and a new absorption maximum at 491 nm is obtained. The dissociation constant, K_D, for the methylamine dehydrogenase–NH_4^+ complex was found to be 16 mM. Anaerobic reductive titration of the enzyme–NH_4^+ complex proceeds through a semiquinone intermediary to a dihydroquinone and requires 2 mol of reductant/mol of enzyme. Although the spectra of the latter two forms of the enzyme–NH_4^+ complex appear very similar to that obtained in the reductive titration of NH_4^+-free enzyme, ESR spectra and reactivity toward O_2 implicate the association of NH_4^+ with the reduced enzyme. At V_{max}, NH_4^+ causes a 2-fold increase in enzyme activity, although during turnover, the enzyme is gradually inactivated. Formaldehyde itself does not perturb the spectral properties of the enzyme; however, in the presence of NH_4^+, a spectrum very similar to that of reduced enzyme is obtained. On titration of the enzyme with 2-bromoethylamine, the changes in absorbance suggest that the enzyme undergoes a two-electron reduction followed by comproportionation of the electrons into the two cofactors on the native enzyme. After treatment with this amine, the enzyme is inactive, suggesting that the reactive product, α-bromoacetaldehyde, has modified the enzyme. Semicarbazide, hydrazine, and hydroxylamine each cause spectral changes in the visible absorbance of the enzyme and bind essentially quantitatively in a molar ratio of 1:1 to the enzyme. The resulting adducts lack enzymatic activity.

In recent years, a new class of bacterial dehydrogenases has been discovered. These enzymes have been named quinoproteins because they contain the novel quinone cofactor methoxatin (Salisbury et al., 1979), alternatively called pyrroloquinoline quinone (Duine et al., 1980). The cofactor has been obtained from methanol dehydrogenases isolated from *Hypomicrobium* X and from *Methylophilis methylotropis*, and its structure, determined by NMR[1] and mass spectral data, is 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione (Duine et al., 1980):



This structure has since been confirmed by organic syntheses in two different laboratories (Corey & Tramontano, 1981; Gainor & Weinreb, 1982).

In additional to methanol dehydrogenase, this cofactor is found in other alcohol dehydrogenases, a glucose dehydrogenase, a lactate dehydrogenase, methylamine dehydrogenases, and other proteins with as yet unknown functions (Duine & Frank, 1981). The cofactor for all these proteins is noncovalently bound except for methylamine dehydrogenase (EC 1.4.99.3). The evidence for a methoxatin type cofactor in the latter enzyme comes from a comparison of the ESR and ENDOR spectra of the semiquinone radicals of both methylamine and methanol dehydrogenases (De Beer et al., 1980). The site of attachment on the cofactor and the aminoacyl residue involved in the linkage have not been identified as yet.

We became involved with these quinoproteins because of our interest in the metabolism of one-carbon compounds. Bacterium W3A1, under various conditions, produces trimethylamine dehydrogenase, methylamine dehydrogenase, and methanol dehydrogenase. The first enzyme contains FMN covalently attached to the protein (Steenkamp et al., 1978),

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[1] Abbreviations: *Ps.*, *Pseudomonas*; PMS, phenazine methosulfate; ESR, electron spin resonance; DCIP, 2,6-dichloroindophenol; NMR, nuclear magnetic resonance; ENDOR, electron-nuclear double resonance; $NaDodSO_4$, sodium dodecyl sulfate.

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and this laboratory has had a long-standing interest in the characterization of enzymes containing covalently bound cofactors (Singer & Kenney, 1974; Edmondson & Singer, 1976; Kenney, 1980). It was therefore logical that we extended our studies to proteins containing other unique oxidation–reduction centers such as the quinoproteins and, in particular, methylamine dehydrogenase since it contains an unusual covalently bound coenzyme.

This paper presents novel spectral properties and information on the oxidation–reduction properties of the enzyme. In addition, evidence is presented for the interaction of this enzyme with ammonium ion and amino-containing carbonyl reagents. The effect of formaldehyde in the presence of ammonium ion, both products of the reaction catalyzed by the enzyme, is considered. A comparison of several properties of methylamine dehydrogenase, which we have isolated from bacterium W3A1 with similar enzymes isolated by others from different bacteria (Eady & Large, 1971; Shirai et al., 1978; Matsumoto, 1978; Matsumoto & Tobari, 1978), suggests that they are all members of an unusual class of oxidation–reduction enzymes.

Materials and Methods

Materials. Hydrazine sulfate, hydroxylamine hydrochloride, semicarbazide hydrochloride, and ammonium sulfate were reagent grade. 2-Bromoethylamine hydrobromide (99%) was obtained from Aldrich Chemical Co., Milwaukee, WI, and was used without further purification. Paraformaldehyde was from Eastman Kodak Co., Rochester, NY.

Reagent-grade glucose oxidase was obtained from Miles Laboratories, Inc., Elkhart, IN. Beef liver catalase, rabbit muscle lactate dehydrogenase, bovine hemoglobin, and beef heart cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, MO. Molecular weight markers (14 300–71 500 range) for $NaDodSO_4$–polyacrylamide gel electrophoresis were from Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, NY.

Bio-Gel A-0.5m (100–200 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA, DEAE-cellulose was from Whatman, Inc., Clifton, NJ, and CM-Sephadex (C-50) was from Pharmacia Fine Chemicals, Piscataway, NJ.

Preparation of Methylamine Dehydrogenase. In our previous studies on trimethylamine dehydrogenase from bacterium W3A1, we found that methylamine dehydrogenase was also formed by this organism when grown on trimethylamine. Thus, the growth of the organism and the initial steps of purification followed that reported by Steenkamp & Mallinson (1976). All operations of the purification procedure were performed at 4 °C. Methylamine dehydrogenase did not bind to DEAE-cellulose column, and it was this step which separated the enzyme from trimethylamine dehydrogenase. To the fraction containing methylamine dehydrogenase was added 1 mg of calcium phosphate gel/mg of protein (Singer & Kearney, 1950). After the fraction was stirred for 15 min, the gel was collected by centrifugation at 1200*g* and then washed with 25 mM potassium phosphate, pH 7.0. Methylamine dehydrogenase was eluted from the gel with two extractions of 0.4 M potassium phosphate, pH 7.2. The combined extracts were brought to 85% saturation in ammonium sulfate (55.9 g/100 mL). After the extracts were allowed to stand for 1 h, the precipitate was collected by centrifugation at 24000*g* for 20 min, dissolved in water, and dialyzed against 25 volumes of water and then 25 volumes of 10 mM potassium phosphate, pH 7.2. After dialysis, the enzyme solution was centrifuged as described above to remove a small amount of insoluble material and then applied to a column of CM-Sephadex C-50 equilibrated with 10 mM potassium phosphate, pH 7.2. For 2000 mg of protein, a column size of 2.5 × 21.5 cm was used. The column was washed extensively with the equilibration buffer and the enzyme then eluted by using a 1-L linear gradient of 10–100 mM potassium phosphate, pH 7.2. Those fractions containing methylamine dehydrogenase activity were pooled and concentrated by ultrafiltration over an Amicon PM-30 membrane. One-fourth volume of ethylene glycol was added and the enzyme stored at −20 °C until used. The enzyme was occasionally stored at this temperature in 5 mM potassium pyrophosphate, pH 7.7.

Molecular Weight of Methylamine Dehydrogenase. The molecular weight of the enzyme was determined by gel filtration on a Bio-Gel A-0.5m column (1 × 80 cm) equilibrated with 50 mM sodium phosphate and 0.5 M sodium chloride, pH 7.0. The marker proteins used (and their molecular weights) were catalase (222 000), lactate dehydrogenase (134 000), hemoglobin (64 500), and cytochrome *c* (13 400). The latter two proteins were monitored by their absorbance at 405 nm. Catalase activity was estimated from the decrease in absorbance at 235 nm on incubation of the enzyme with 10 mM H_2O_2 in 0.067 M sodium pyrophosphate, pH 7.7. Lactate dehydrogenase activity was measured at 340 nm in 0.1 M sodium pyrophosphate, pH 7.7, containing 0.5 mM sodium pyruvate and 0.15 mM NADH.

The molecular weights of the subunits of methylamine dehydrogenase were determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

Resolution of the Peptides of Methylamine Dehydrogenase. The enzyme (49 mg) was dialyzed against water at 4 °C until it was essentially salt free. Urea was added at room temperature to give a final concentration of 8 M. After being allowed to stand for 1 h, the solution was applied to a column (0.9 × 6 cm) of DEAE-cellulose (acetate form) equilibrated with freshly prepared 8 M urea. The column was then washed successively with 2 column volumes each of 8 M urea, water, and 0.01 M acetic acid. The chromophoric subunit, which originally bound to the DEAE-cellulose, was then eluted with 0.05 M acetic acid.

Titrations. Anaerobic titrations with sodium dithionite were performed as described by Edmondson & Singer (1973) at room temperature in 0.1 M sodium pyrophosphate, pH 7.7 or pH 9.0. The dithionite solutions were standardized against an anaerobic riboflavin solution of known concentration. The enzyme solutions were made anaerobic by careful evacuation and flushing the cuvette 6 times with O_2-free Ar. Methylamine hydrochloride solutions were made anaerobic as follows. Into a test tube was added 0.36 mL each of 0.1 M glucose and 0.1 M sodium acetate, pH 5.1. Sufficient water was added so that the final volume, after addition of all components, was 3.6 mL. After the air above the solution was replaced with Ar, the tube was stoppered with an air-tight rubber septum. The tube was shaken briefly to allow dissolved gases to exchange with the vapor phase. The latter was replaced with fresh Ar by using disposable syringe needles as an inlet and an outlet for the gas. This procedure was repeated 4 times, and then 20 units of glucose oxidase (1000 units/mL), 25 units of catalase (8000 units/mL), and a sufficient quantity of 0.1 M methylamine hydrochloride to give the desired final concentration (2–10 mM) were added by syringe. The enzyme was titrated with this solution anaerobically in a manner similar to the dithionite titrations at ambient temperature in 0.1 M sodium pyrophosphate, pH 7.7 or pH 9.0.

Methylamine dehydrogenase was titrated aerobically at room temperature with ammonium sulfate solutions in 0.1 M sodium pyrophosphate, pH 7.7 or pH 9.0, and with semi-

carbazide hydrochloride, hydrazine sulfate, and hydroxylamine hydrochloride in 100 mM sodium pyrophosphate, pH 7.7.

Production of Formaldehyde. Paraformaldehyde, 0.6 g, was placed in a Pyrex tube with 10 mL of water. The tube was sealed and incubated at 100 °C for 4 h. After the tube was cooled, the contents were removed and diluted to 0.8 M in formaldehyde with the buffer used for a given experiment.

Reaction of 2-Bromoethylamine with Methylamine Dehydrogenase. This reaction was followed under both aerobic and anaerobic conditions. The anaerobic solution of 0.9 mM 2-bromoethylamine was prepared in a test tube fitted with an air-tight septum. The contents were flushed 6 times with oxygen-free argon. The enzyme was titrated with this solution as described above for dithionite solutions.

Evaluation of Data. Computer analysis of the nonlinear data involved the linear jackknife technique described by Fox et al. (1980) with logarithmic transformation of the parameters (Oppenheimer et al., 1981). The program incorporated the DUD nonlinear least-squares algorithm (Ralston & Jennrich, 1978) from which the value of the sum of the squared residuals was obtained. Linear regression analysis was also performed by computer. Programs were written in Basic and run on a Sinclair ZX 81 minicomputer (Sinclair Research Ltd., Boston, MA), equipped with a 64-kilobyte memory board (Memotech Ltd., Oxford, England). Note that the logarithmic transformations occasionally generate unequal upper and lower values for the standard errors (67% confidence region).

The equation used for the analysis of the binding of NH_4^+ to methylamine dehydrogenase was based on a modified Benesi–Hildebrand plot (Benesi & Hildebrand, 1949) in which the concentration of NH_4^+ is substituted for the concentration of H^+. Thus

$$\Delta A = \frac{\Delta A_{max}[S]}{K_D + [S]} \quad (1)$$

which can be rearranged to give

$$\frac{1}{\Delta A} = \frac{K_D}{\Delta A_{max}} \frac{1}{[S]} + \frac{1}{\Delta A_{max}} \quad (2)$$

where K_D is the dissociation constant for the NH_4^+–enzyme complex, S is NH_4^+, and ΔA is the absorbance difference between that obtained on the addition of NH_4^+ and that of the NH_4^+-free enzyme. Equation 1 was used for the computer analyses, and eq 2 was used for the graphic presentation where the ordinate intercept in $1/\Delta A_{max}$ and the abscissa intercept is $-1/K_D$.

Other Methods. Steady-state enzyme assays, using the PMS/DCIP couple, were performed as described by Eady & Large (1968) at 30 °C in 0.1 M sodium pyrophosphate, pH 7.7. Single-point analyses were performed at 2 mM PMS and 3.3 mM methylamine hydrochloride. Absorption spectra were recorded on a Cary 14 spectrophotometer interfaced with a 2/4 Nova computer (On-Line Instrument Systems, Athens, GA) or a Cary 219 spectrophotometer. ESR spectra were obtained on a Varian E-4 spectrometer at room temperature. Fluorescence measurements were made on a Perkin-Elmer MPF-3 fluorescence spectrophotometer.

Protein concentrations were measured by the biuret method (Gornall et al., 1949) with bovine serum albumin as standard. For the cofactor-containing peptide, the absorbance at 540 nm was corrected for contribution of the cofactor to the absorbance at that wavelength. From the purified enzyme, a value of $\epsilon_{278nm} = 0.92$ cm^{-1} (mg/mL)$^{-1}$ was calculated and used to estimate methylamine dehydrogenase concentrations.

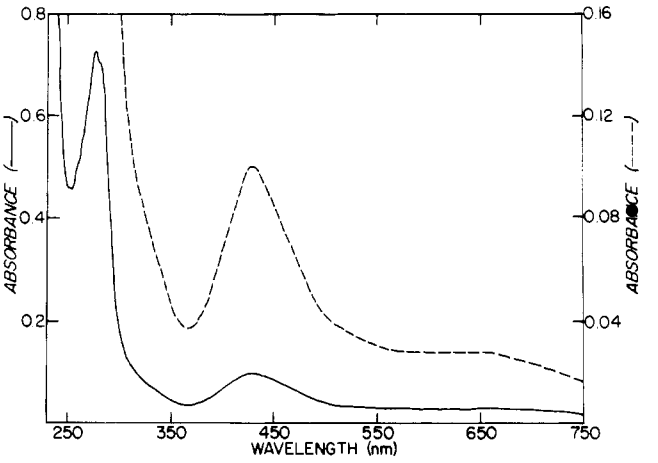


FIGURE 1: Absorption spectrum of methylamine dehydrogenase from bacterium W3A1. The enzyme (0.78 mg/mL) was in 0.1 M sodium pyrophosphate, pH 7.7, at room temperature. The dashed line represents a 5-fold increase of the absorbance scale.

Results

Purification of Methylamine Dehydrogenase. When grown on trimethylamine as the carbon source, bacterium W3A1 produces methylamine dehydrogenase in addition to trimethylamine dehydrogenase. The former enzyme is also produced when methylamine is the sole carbon source. Indeed, over 4 times as much of this enzyme is formed when this carbon source is used, and the enzyme accounts for approximately 15% of the protein in the cellular extract. The organism is also devoid of a slimelike encapsule, the lack of which facilitates harvesting of the organism and release of the enzyme in the initial stages of purification. Since the enzyme constitutes such a large fraction of the extractable protein, it is quite easy to purify. Following the procedure outlined under Materials and Methods, when grown on trimethylamine, the enzyme is obtained in homogeneous form with a yield of approximately 50%. In addition, trimethylamine dehydrogenase and methanol dehydrogenase[2] are also obtained. With methylamine as the carbon source during growth, the latter two enzymes are absent, although preliminary results suggest that a protein with spectral properties corresponding to methanol dehydrogenase is formed.[2]

Physical Properties of Methylamine Dehydrogenase. In addition to the characteristic protein absorbance maximum at 278 nm, the enzyme absorbs light in the visible region with a maximum at 429 nm (Figure 1). As will be shown, this latter maximum is affected by substrate, reductants, and other amino-containing compounds.

The molecular weight of this enzyme, as determined by gel chromatography on Bio-Gel A-0.5m, was found to be 127 000 ± 5000. $NaDodSO_4$–polyacrylamide gel electrophoresis yields two polypeptides of M_r 45 000 and 15 500. These data suggest that the enzyme is composed of two molecules each of the larger and smaller polypeptides, i.e., an A_2B_2 type structure.

Evidence for Covalent Linkage of the Cofactor to the Smaller Subunit. Chromatography on DEAE-cellulose of methylamine dehydrogenase in 8 M urea yields two polypeptide bands. The smaller peptide has absorbance maxima at 277 and 424 nm and thus contains the cofactor. The cofactor also migrates with the M_r 15 500 subunit on $NaDodSO_4$–polyacrylamide gel electrophoresis.

Anaerobic Reductive Titrations of Methylamine De-

[2] W. C. Kenney and W. McIntire, unpublished results.

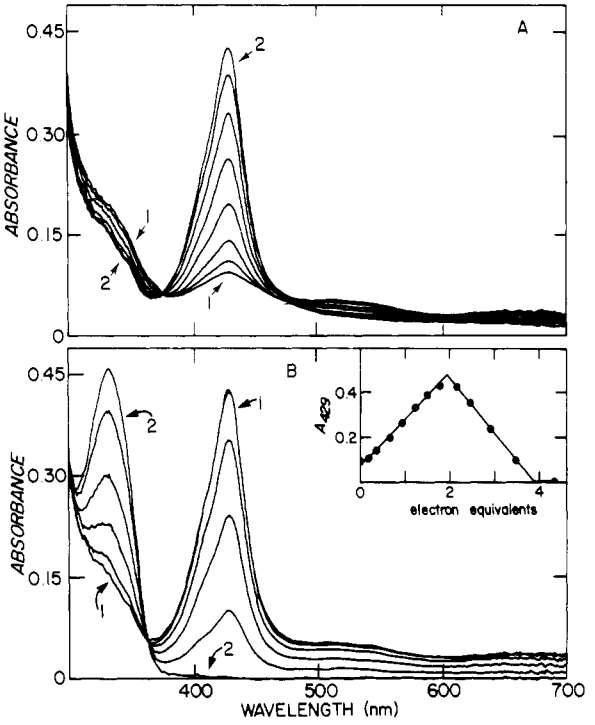


FIGURE 2: Dithionite titration of methylamine dehydrogenase. The enzyme (1.57 mg/mL) was in 2.4 mL of 0.1 M sodium pyrophosphate, pH 9.0. (A) First phase of the titration. Curve 1 is the spectrum of the oxidized enzyme. The spectra, in increasing order of absorbance at 429 nm, were taken after the anaerobic addition of 2.79, 5.58, 9.76, 13.9, 18.1, 25.1, and 26.5 (curve 2) nmol of dithionite. Spectra were recorded 5–20 min after addition of reductant to allow the end point to be reached. (B) Second phase of the titration. Curve 1 in part B corresponds to curve 2 in part A. Continuing from part A, subsequent additions of dithionite were 32.1, 36.2, 46.7, 51.6, and 64.1 nmol. Note that the spectrum taken after the addition of 32.1 nmol of dithionite has nearly the same absorbance at 429 nm as for curve 1. Differences, however, are apparent at 330 and 490 nm. Curve 2 in part B is the spectrum of the fully reduced enzyme. Spectra were recorded 20–40 min after each addition of dithionite. The inset to (B) is a graph of the absorbance at 429 nm vs. the electron equivalents of dithionite per mol of enzyme (assumed molecular weight of 127 000). All spectra in (A) and (B) are corrected for volume changes to 2.4 mL.

hydrogenase. Some of the more interesting features of this enzyme are the absorbance changes which occur on incubating the enzyme with methylamine or the reductant, sodium dithionite. Titration of methylamine dehydrogenase with the latter compound is shown in Figure 2. Curve 1 of Figure 2A is the absorbance spectrum of the oxidized enzyme, and curve 2 is the spectrum obtained after the addition of 1.9 electron equiv of dithionite/mol of enzyme. (In this and subsequent calculations, a molecular weight of 127 000 is assumed.) At pH 9.0, this phase of the reduction results in a 4.5-fold increase in the absorbance at 429 nm. The second phase of the titration (Figure 2B), which consumes 2.0 electron equiv of dithionite/mol of enzyme, similarly produces dramatic spectral changes. The absorbance at 429 nm becomes nearly zero, and simultaneously an absorbance maximum at 331 nm appears (3-fold increase in absorbance). The isosbestic points at 375 nm in the first phase and at 364 nm in the second phase indicate that well-behaved single reactions take place in each phase of the titration. Similar titration curves and stoichiometry were obtained for the titration at pH 7.7. Thus, the first and second phases of the titration at this pH were isosbestic at 378 and 364 nm, respectively. It should be noted, however, that the absorbance increase in the first (429 nm) and the second (331 nm) phases were smaller at pH 7.7 than

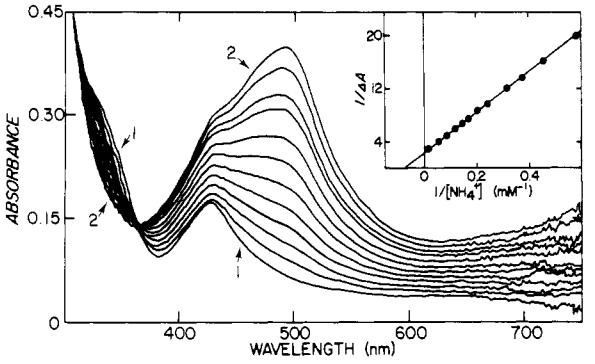


FIGURE 3: Ammonium ion titration of methylamine dehydrogenase. The enzyme (2.51 mg/mL) was in 0.1 M sodium pyrophosphate, pH 9.0, at room temperature. Curve 1 is the oxidized enzyme in the absence of NH_4^+. For the subsequent spectra, with increasing absorbance at 491 nm, additions of NH_4^+ were made to give final concentrations of 0.74, 1.72, 2.68, 4.11, 5.98, 8.71, 12.3, 14.0, 23.0, 40.9, and 66.5 mM. Curve 2 is the spectrum of methylamine dehydrogenase at the latter concentration of NH_4^+. All spectra were corrected to 2.0 mL for volume changes due to addition of aliquots of ammonium sulfate. (Inset) Plot of $1/\Delta A_{491}$ vs. $1/[NH_4^+]$. The data from this figure and from spectra at intermediary concentrations of NH_4^+ are used for this plot. ΔA_{491} is the absorbance observed at a given concentration of NH_4^+ minus the absorbance of the untreated enzyme.

at pH 9.0 (2.2- and 2.7-fold change, respectively). Comparison of the initial, intermediate, and final absorbance spectra (cf. curves 1 and 2 of Figure 2A,B) with the corresponding spectra obtained at pH 7.7 indicates that the differences in the change in absorbance at 429 and 331 nm, seen at the two pH values, are primarily a result of higher initial absorbance at pH 7.7, for the absorbances at 429 and 331 nm.

The enzyme was also titrated at pH 7.7 with methylamine, the natural substrate. The titration curves (not shown) were nearly identical with those obtained anaerobically with dithionite at this pH, and the isosbestic points occurred at the same wavelengths (378 and 364 nm). As in the dithionite titrations (see legend to Figure 2), a period of 10–20 min after addition of reductant in the first phase and 20–45 min in the second phase was necessary for spectral changes to be completed. The titration required 1.9 molar equiv of methylamine/mol of enzyme for complete reduction.

The rate of reoxidation of the fully reduced enzyme in air-saturated buffer was so slow that, even after several days at 4 °C, only a small percentage of the enzyme was reoxidized (cf. curve 2 of Figure 2B). When the enzyme was reduced only to the end of the first phase at pH 7.7, and the solution then saturated with air by careful shaking, the reoxidation proceeded slowly [calculated rate constant = $(14.6 \pm 0.3) \times 10^{-3}$ min^{-1}]. The final spectrum then obtained was identical with the spectrum of enzyme taken prior to treatment with reductant.

Effect of Ammonium Ion on Methylamine Dehydrogenase. During purification of methylamine dehydrogenase, ammonium sulfate precipitation was sometimes used to concentrate the enzyme from dilute solutions. In this procedure, it was observed that the enzyme solution turned from a greenish to a reddish hue. Since NH_4^+ is one of the products of methylamine oxidation by the enzyme (Eady & Large, 1968), experiments were undertaken to investigate the role of NH_4^+ in bringing about this color change. Figure 3 presents the spectral changes which the enzyme undergoes on titration with ammonium sulfate at pH 9.0. The reason that there are color changes, as mentioned above, becomes apparent from this series of spectra. Large increases in absorbance are seen from

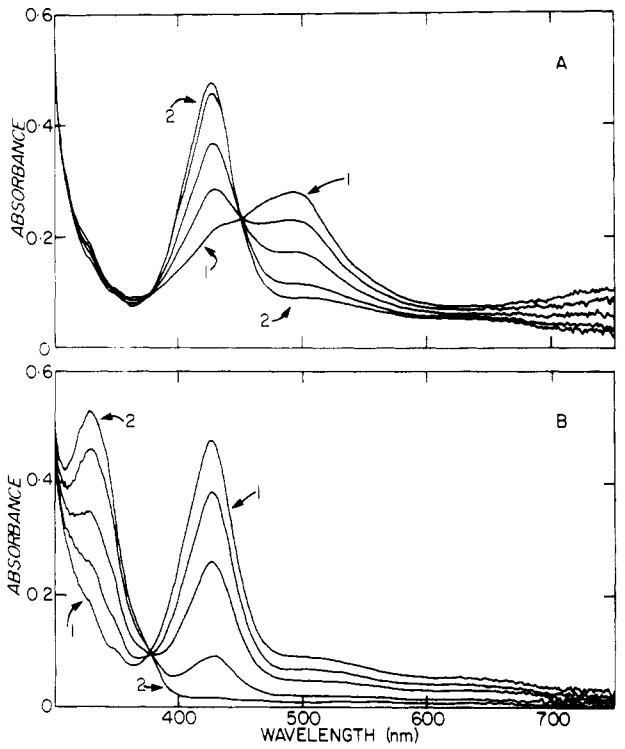


FIGURE 4: Dithionite reduction of methylamine dehydrogenase in the presence of ammonium ion. The enzyme (5.02 mg, 39.5 nmol) was in 2.4 mL of 0.1 M sodium pyrophosphate, pH 9.0, containing 41 mM $(NH_4)_2SO_4$. (A) Curve 1 is the oxidized enzyme–NH_4^+ complex. Subsequent spectra, with decreasing absorbance at 491 nm, were taken after the addition of 6.6, 13.2, 21.5, and 26.5 nmol of sodium dithionite. (B) Curve 1 corresponds to curve 2 of Part A. Subsequent spectra, with decreasing absorbance at 427 nm, were taken after the addition of 36.4, 46.4, 59.6, and 76.2 nmol (curve 2) of reductant. All spectra are corrected to 2.4 mL volume.

365 to 750 nm as ammonium sulfate is added to the enzyme, and the spectra are isosbestic at 364 nm. Maximum changes occur at 491 nm, and there is an indication for a new absorbance maximum in the near-infrared (>750 nm). The inset to Figure 3 presents a double-reciprocal plot, according to eq 2, of the absorbance changes occurring at 491 nm. The value of ΔA_{max} is 0.155 mg^{-1} mL^{-1} and the K_D value is 11.5 ± 0.1 mM. Similar titrations, performed at pH 7.7 (not shown), give a K_D value of 15.4 ± 0.5 mM. In the calculations of these constants, the pK_a value for NH_4^+ was not considered; that is, in Figure 3, the total of NH_4^+ plus NH_3 was included. Comparison of the K_D values at pH 7.7 with those at pH 9.0 indicates that it is NH_4^+ which interacts with the enzyme rather than NH_3. The concentration of the latter species increases 10-fold between pH 7.7 and pH 9.0, and a comparable change in K_D would be expected if NH_3 were the interacting species. On the other hand, the increase in K_D value from pH 9.0 to pH 7.7 is in line with the relative increase in NH_4^+ between these pH values. The presence of an ionizing group on the enzyme itself cannot be ruled out, however, as an explanation for these absorbance changes.

The effect of NH_4^+ on the oxidation–reduction properties of the enzyme was also investigated. In Figure 4, the dithionite titration at pH 9.0 of the methylamine dehydrogenase–NH_4^+ complex is presented. As in titrations in the absence of NH_4^+ (Figure 2), the spectral changes are biphasic. Concomitant with the loss in absorbance at 491 nm, there is an increase in absorbance at 427 nm on addition of dithionite (Figure 4A). The spectrum at the end of the first phase (Figure 4A, curve 2) is very similar to that obtained in the absence of NH_4^+. Subsequent addition of dithionite leads to bleaching of the absorbance at 427 nm with a simultaneous increase in absorbance at 330 nm, as was observed previously in titrations in the absence of NH_4^+. For each addition of reductant, spectral changes ceased within 5–10 min during each phase of the titration, in contrast to a waiting period of up to 20 min in the first phase and 45 min in the second phase when NH_4^+ was excluded. Another significant difference between the dithionite titrations in the presence and absence of NH_4^+ was the rates of reoxidation of both phases in air-saturated buffer. In the presence of NH_4^+, the fully reduced form of the enzyme (curve 2 of Figure 4B) reoxidized to the intermediate form in less than 30 min (rate constant of 0.103 ± 0.005 min^{-1}). In titrations without added NH_4^+, the fully reduced enzyme was essentially resistant to reoxidation in air. The intermediate obtained at the end of the first phase of titration (curve 2 of Figure 4A) was ca. 90% reoxidized after 16 h in air-saturated buffer, in contrast to the 3–4 h required for reoxidation in the absence of NH_4^+. In the titration presented in Figure 4, 0.65 mol of dithionite was required in the first phase and 1.02 mol in the second phase per mol of enzyme (assuming a molecular weight of 127 000).

Even though the absorption spectra of the intermediate species and of the fully reduced states of the enzyme are very similar in the dithionite titrations in the presence and absence of NH_4^+, the differences in the reactivity of the various forms of the cofactor with oxygen suggests that there is a specific interaction of NH_4^+ with the reduced forms of the cofactor. Further support for this conclusion is given by a comparison of the ESR of the enzyme radicals in the presence and absence of NH_4^+ (Figure 5). The important feature is the line width of the radical in the presence of NH_4^+, 11 G, vs. that in the absence of NH_4^+, 7.5 G. The g values are 2.0075 and 2.0086, respectively.

The influence of NH_4^+ is also evident in the steady-state kinetics of the enzyme. NH_4^+ appears to act as a classical, reversible, competitive inhibitor for methylamine. The data were subjected to computer analyses by using the equation

$$v = \frac{V_{max}}{[MA] + K_{MA}(1 + [NH_4^+]/K_D)} \quad (3)$$

where v is the initial rate of reaction, V_{max} is the maximum rate, K_{MA} is the Michaelis constant for methylamine, MA, and K_D is the dissociation constant of the enzyme–NH_4^+ complex. A summary of the values obtained is given in Table I. Unexpectedly, NH_4^+ increases the values of both V_{max} and K_{MA} when compared to the steady-state assays performed in the absence of NH_4^+.

In the steady-state assays of the methylamine dehydrogenase activity in the presence of NH_4^+, the absorbance change of DCIP does not decrease linearly, but rather the rate rapidly decreases to zero. This time-dependent inactivation behaves in a pseudo-first-order manner. This is in contrast to assays in the absence of NH_4^+ in which the initial rates are linear and all the terminal electron acceptor is reduced when excess amounts of methylamine and PMS are present. As indicated in Table II, as the concentration of added NH_4^+ increases, the rate of inhibition also increases, again suggesting an interaction between this cation and the enzyme. At high methylamine concentration, the initial rate is unchanged, regardless of the NH_4^+ concentration (Table II), indicating that methylamine saturates the active site and NH_4^+ cannot compete effectively for this site. Hence, the observed first-order inhibition could be a result of NH_4^+ binding at other than substrate binding domain. A possible mechanism is given as follows:

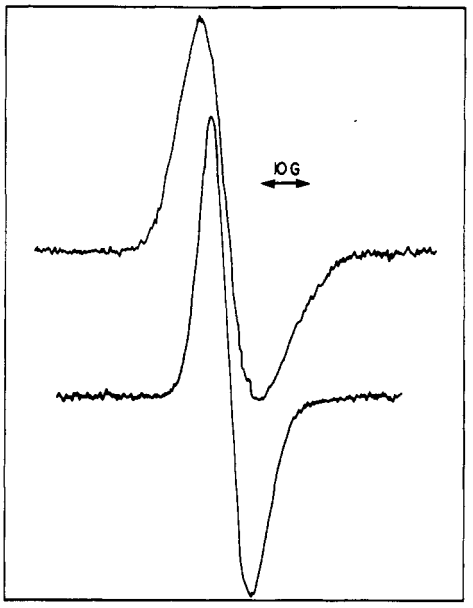


FIGURE 5: ESR spectra of methylamine dehydrogenase radical in the presence or absence of ammonium ion. Upper spectrum: The enzyme (5.77 mg, 45.4 nmol) in 0.5 mL of 0.05 M sodium pyrophosphate, pH 9.0, containing 40 mM $(NH_4)_2SO_4$ was mixed anaerobically with 50 μL of 1.33 M sodium dithionite (66.5 nmol). After standing 30 min to allow for reduction of enzyme, the solution was exposed to air, and the ESR spectrum of the stable radical was taken. Spectrometer settings were the following: time constant 3 s; modulation amplitude 2 G; receiver gain 3.2×10^3; microwave frequency 9.084 GHz; microwave power 4.5 mW; modulation frequency 10 GHz. Lower spectrum: The enzyme (1.88 mg, 14.8 nmol) in 0.15 mL of 0.05 M sodium pyrophosphate, pH 9.0, was mixed anaerobically with 52.2 nmol of sodium dithionite in 0.036 mL of buffer. The spectrum was recorded at room temperature 3.5 min after mixing. The spectrometer parameters were the following: modulation frequency 100 KHz; modulation amplitude 2 G; microwave power 0.75 mW; microwave frequency, 9.106 GHz. Note that the quantity of radical in the two experiments cannot be compared because of different ESR sample holder geometry and volume and because of the different microwave power settings.

$$E + NH_4^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}NH_4^+ \xrightarrow{k_2} E^*{\cdot}NH_4^+ \qquad (4)$$

E* is an inactive form of the enzyme, and E is the form of the enzyme which is susceptible to this type of inactivation. According to Strickland et al. (1975), when k_{-1} is much greater than k_2, the steady-state equation for the observed rate constant for the loss in activity can be expressed as

$$k_{obsd} = \frac{k_2[NH_4^+]}{[NH_4^+] + K_D} \qquad K_D = \frac{k_{-1}}{k_1} \qquad (5)$$

Computer analysis of the data given in Table II yields a value for $k_2 = 0.21 \pm 0.02$ min^{-1} and for $K_D = 14.7 \pm 2.9$ mM. The latter value is in good agreement with that determined by spectral and kinetic analyses (compare above). The data in Table II also indicate that, as the concentration of NH_4^+ increases, the amount of DCIP (i.e., PMS) that is consumed decreases. From these values, it can be estimated that, at infinite NH_4^+ concentration and for the conditions described in Table II (e.g., 3.1 mM methylamine), the enzyme turns over approximately 14 times prior to being inactivated.

As is expected from eq 5, k_{obsd} is independent of enzyme concentration. The absolute amount of DCIP consumed prior to inactivation is, however, proportional to enzyme concentration. Results comparable to the above assays, which were performed in air-saturated solutions, were obtained with oxygen-saturated and argon-saturated assay solutions, suggesting that oxygen does not play a role in the NH_4^+-induced inactivation during turnover of the enzyme.

Table I: Steady-State Kinetic Constants for Methylamine Dehydrogenase in the Presence and Absence of Ammonium Ion[a]

ammonion ion	V_{max} (μmol/min·mg)	K_{MA} (μM)	K_D for NH_4^+ (mM)
absent	$11.1^{+0.1}_{-0.2}$	16.4 ± 0.5	
present	$23.0^{+1.6}_{-1.5}$	$37.8^{+6.2}_{-5.3}$	$16.6^{+3.9}_{-3.1}$

[a] Enzymic assays were performed at 30 °C in 0.1 M sodium pyrophosphate, pH 7.7, containing 2 mM PMS. Methylamine concentrations were at 7.8, 15.6, 31.2, or 78 μM. In those assays containing NH_4^+, the concentrations of this cation were at 2.67, 6.67, 20, or 33.3 mM.

Table II: Ammonium Ion Inactivation in the Steady-State Reaction Catalyzed by Methylamine Dehydrogenase[a]

$[NH_4^+]$ (mM)	k_{obsd}[b] for enzyme inactivation (min^{-1})	$\Delta DCIP_{total}$[b] (μmol/mg)	$V_{initial}$ (μmol/min·mg)
4.0	0.319 ± 0.006	59.9 ± 1.2	19.1 ± 0.1
8.0	0.592 ± 0.012	31.2 ± 0.4	18.5 ± 0.1
16.7	1.03 ± 0.03	19.9 ± 0.4	20.5 ± 0.2
33.3	1.56 ± 0.03	14.1 ± 0.1	22.0 ± 0.2
66.7	1.92 ± 0.06	10.5 ± 0.1	20.1 ± 0.4
133.0	1.95 ± 0.07	10.2 ± 0.2	19.9 ± 0.4

[a] Assays were performed at 30 °C in 0.1 M sodium pyrophosphate, pH 7.7, 2 mM PMS, and 3.12 mM methylamine. [b] $\Delta DCIP_{total}$ and k_{obsd} are calculated by jackknife fit to the equation $\Delta DCIP = \Delta DCIP_{total}e^{-k_{obsd}t}$. Integration of this equation with respect to t gives $v_{initial}$, the rate at $t = 0$, which is the product of k_{obsd} times $\Delta DCIP_{total}$.

Effect of Formaldehyde on Methylamine Dehydrogenase. In the absence of NH_4^+, formaldehyde has no effect on the spectral properties of methylamine dehydrogenase. On the other hand, if formaldehyde is added to the enzyme–NH_4^+ complex, the absorbance maximum at 491 nm associated with this complex disappears (Figure 6), and the resulting spectrum is similar to that obtained at the end of the second phase of the dithionite titration (absorbance maxima at 424 and 324 nm). The latter peak at 324 nm is particularly indicative of the second phase of the reductive titration. The reduced type spectrum seen in Figure 6 suggested that formaldehyde may be acting as a substrate. We were unable, however, to demonstrate the reduction of PMS by formaldehyde, either in the presence or in the absence of NH_4^+. The absorbance changes at 491 and 324 nm occur rather slowly when formaldehyde is incubated with the enzyme–NH_4^+ complex. The observed pseudo-first-order rate constant is 0.133 ± 0.001 min^{-1} at 0.48 mM formaldehyde, 67 mM NH_4^+, and 40 μM methylamine dehydrogenase.

Effect of 2-Bromoethylamine on Methylamine Dehydrogenase. The specificity of methylamine dehydrogenase for *n*-alkylamines is very broad (Eady & Large, 1968; Matsumoto, 1978), and it might be expected that 2-bromoethylamine would also be oxidized by the enzyme. Our reason for considering this compound is based on its inhibitory effect of another amine-oxidizing enzyme, i.e., plasma amine oxidase (Suva & Abeles, 1978). For the latter enzyme, 2-bromoethylamine was found to be a suicide inhibitor because oxidase converts this amine to α-bromoacetaldehyde, a compound capable of reacting with several functional groups on the enzyme.

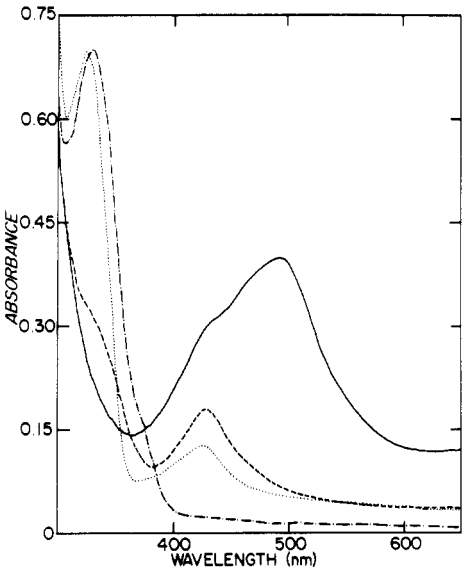


FIGURE 6: Effect of formaldehyde on the spectral properties of methylamine dehydrogenase in the presence and absence of ammonium ion. Spectrum of the oxidized enzyme, 2.51 mg/mL, in 0.1 M sodium pyrophosphate, pH 9.0 (---). Spectrum after the addition of NH_4^+ to 66.5 mM (—). Spectrum taken 1 h after the addition of formaldehyde (final concentration 1.9 mM) to the enzyme–NH_4^+ complex (···). For comparison, a spectrum of the reduced enzyme at the above concentration is included (---). Note that the spectrum obtained on addition of formaldehyde alone to the oxidized enzyme is the same as that of the NH_4^+-free enzyme.

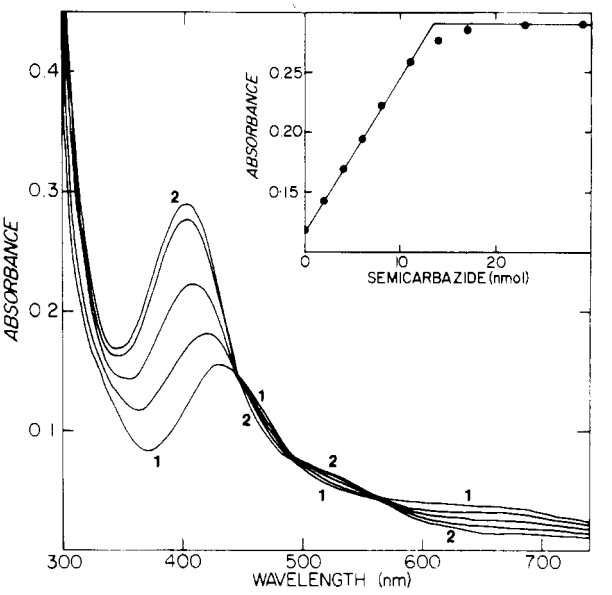


FIGURE 7: Spectral changes of methylamine dehydrogenase induced by semicarbazide. Curve 1 is the spectrum of the untreated enzyme (1.54 mg, 12.1 nmol) in 1 mL of 100 mM sodium pyrophosphate, pH 7.7. Subsequent spectra, with increases in the spectral region around 400 nm, were taken after 4, 8, 14, and 23 nmol (curve 2) of semicarbazide were added. (Inset) A graph of the absorbance change at 400 nm vs. nmol of semicarbazide added. Intermediate spectra used for the points in the inset plot are not included.

On addition of 80 nmol of 2-bromoethylamine to an aerobic solution of 22 nmol of methylamine dehydrogenase in 0.9 mL of 1 M potassium phosphate, pH 7.6, a reduced type enzyme spectrum was initially observed, with a maximum appearing at 324 nm and a 75% decrease in absorbance at 426 nm. This was followed by a slow, time-dependent change in absorbance to a spectrum characteristic of the intermediate form of the enzyme, i.e., increased absorbance at 429 nm and decreased absorbance at 324 nm (cf. curve 1 of Figure 2B). Comparable observations were made when the enzyme was titrated anaerobically with substoichiometric amounts of 2-bromoethylamine, i.e., a rapid increase in absorbance at 325 nm and a decrease in absorbance at 429 nm, indicating that the fully reduced enzyme is formed initially. These changes were followed by a slower decrease of the 325-nm band and an increase in absorbance at 429 nm.

Reaction of Semicarbazide, Hydrazine, and Hydroxylamine with Methylamine Dehydrogenase. Eady & Large (1971) used these compounds to ascertain whether the cofactor of methylamine dehydrogenase from *Ps.* AM1 might be a pyridoxal derivative. These carbonyl reagents did lead to certain spectral changes, and complete inhibition of the enzyme by hydroxylamine was reported (Eady & Large, 1968). On the basis of the chemistry of quinones, these reagents are expected to react with the carbonyl-type carbons of methoxatin as well (Finley, 1974).

In Figure 7, the aerobic titration of methylamine dehydrogenase with semicarbazide is presented. As with NH_4^+, there are dramatic spectral changes. The absorption maximum shifts from 426 to 401 nm, and a well-defined shoulder appears at 520 nm. There are also isosbestic points at 446, 492, and 564 nm. Eady & Large (1971) reported the shift in absorbance maximum but not the relative increase in absorbance. The binding of semicarbazide is tight, as indicated by the plot of absorbance at 400 nm vs. nanomoles of added titrant (inset to Figure 7), and 1.05 mol of semicarbazide/mol of enzyme is required for maximal spectral changes to occur.

When the enzyme was titrated with hydrazine, the absorbance maximum shifted from 426 to 394 nm and the extinction increased 1.3-fold (spectra not shown). Isosbestic points were at 427, 500, and 572 nm, and a shoulder at 410 nm appeared. This titration required 1.08 mol of hydrazine/mol of enzyme to reach the maximal absorbance change.

The spectral data for the titration of methylamine dehydrogenase with hydroxylamine are presented in Figure 8. The interaction again appears specific and results in tight binding. The maximal absorbance undergoes a slight hypsochromic shift to 427 nm and a 2-fold increase over that of the untreated enzyme, a change that is considerably larger than that found by Eady & Large (1971) for reaction of this reagent with methylamine dehydrogenase. For the titration given in Figure 8, 1.06 mol of hydroxylamine/mol of enzyme is required for the observed spectral changes.

Subsequent to these titrations, the enzyme was inactive. Addition of either hydroxylamine, hydrazine, or semicarbazide at the start of an enzyme assay with methylamine as substrate did not change the initial rate but caused a pseudo-first-order inactivation of the enzyme. The apparent rate constant, k_{app}, for this pseudo-first-order inactivation by tight binding inhibitors has been described (Cha, 1975) as

$$k_{app} = \frac{k_1[I]}{1 + [S]/K_{MA}} \quad (6)$$

where I is the inactivator, S is methylamine, K_{MA} is the steady-state constant for methylamine, and k_1 is the rate constant for association of the enzyme–inhibitor complex. Equation 6 can be written as

$$\frac{1}{k_{app}} = \frac{[S]}{K_{MA}k_1[I]} + \frac{1}{k_1[I]} \quad (7)$$

At constant hydroxylamine concentration, a plot of $1/k_{app}$ vs. methylamine concentration gives a straight line with the ordinate intercept equal to $1/(k_1[I])$ and the abscissa intercept

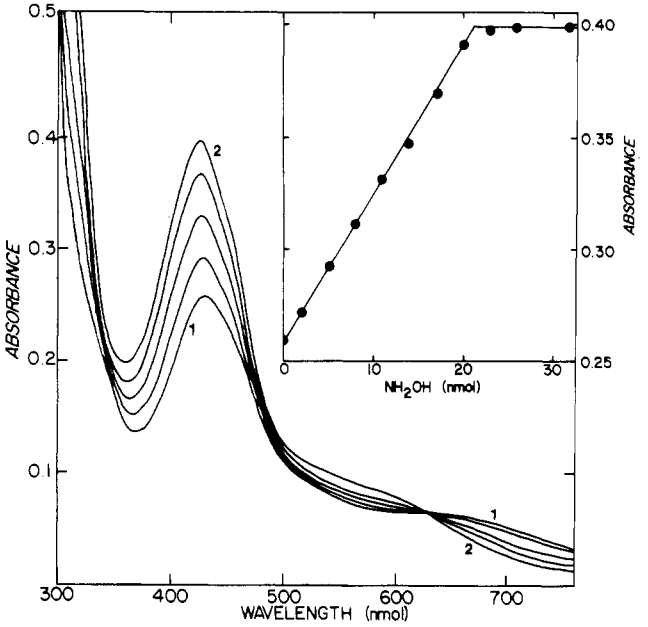


FIGURE 8: Titration of methylamine dehydrogenase with hydroxylamine. Curve 1 is the spectrum of the enzyme (2.52 mg, 19.9 nmol) in 1.0 mL and 100 mM sodium pyrophosphate, pH 7.7. Subsequent spectra, with increasing absorbance at 427 nm, were taken after the addition of 5, 11, 17, and 26 nmol (curve 2) of hydroxylamine. The inset is a graph of the absorbance at 426 nm vs. the nanomoles of hydroxylamine added. Intermediate spectra have not been included for clarity, although their absorbance at 427 nm has been used for the inset graph.

equal to $-1/K_{MA}$. From graphic analyses of k_{app} at various concentrations of methylamine and of hydroxylamine, a value of k_1 equal to 6000 min^{-1} mM^{-1} and a K_{MA} value equal to 18 μM are obtained. The latter value is in good agreement with that reported in Table I.

Dithionite Reduction of the Lower Molecular Weight Subunit of Methylamine Dehydrogenase. It was of interest to compare the oxidation–reduction properties of the cofactor-containing peptide of methylamine dehydrogenase with those of the parent enzyme. Dithionite titration of the cofactor-containing subunit, purified as described under Materials and Methods, was performed, and this titration is shown in Figure 9. The cofactor-containing peptide, as isolated, has an absorption maximum at 424 nm, in addition to a maximum in the ultraviolet at 278 nm. On addition of dithionite, there is a decrease in absorbance at 421 nm with a concomitant increase in absorbance at 306 nm, reminiscent of the absorbance changes observed in the second phase of titration of the native enzyme. On admission of oxygen, however, the absorbance returns to that present initially. One mole of dithionite (two electrons)/per mol of cofactor peptide is required for the changes depicted in Figure 9.

The solution of the cofactor peptide exhibits only very low fluorescence. When excited with light of 280 nm wavelength, the emission maximum is at 390 nm (data not shown). On anaerobic reduction of the peptide with dithionite, the emission intensity increases more than 10-fold and is centered at 410 nm (Figure 10). The excitation maximum is at 322 nm rather than 280 nm, indicating that tryptophan is not responsible for the fluorescent species, but more likely the cofactor which, on reduction, absorbs light at this wavelength (Figure 9). Moreover, the emission maximum is not characteristic of tryptophan-containing peptides. On the other hand, the shoulder at 280 nm in the excitation spectrum is possibly due to energy transfer from tryptophan to the cofactor, although

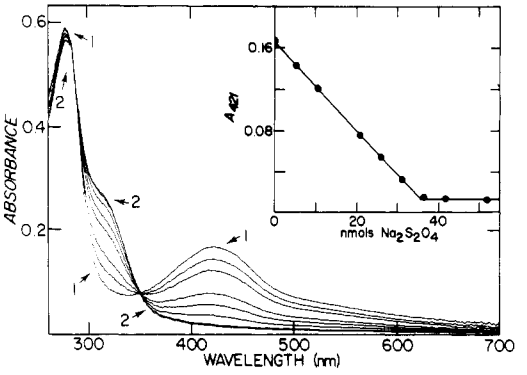


FIGURE 9: Dithionite reduction of the cofactor subunit of methylamine dehydrogenase. The cofactor peptide, 0.5 mg (32.2 nmol), in 2.4 mL of 57 mM sodium pyrophosphate, pH 7.7, has an absorbance spectrum given by curve 1. The subsequent spectra were taken after the addition of 5.2, 10.4, 20.8, 26.0, 31.2, 36.4, 41.6, and 52 nmol of dithionite. All spectra were corrected for dilution. The inset is the decrease in absorbance at 422 nm with added dithionite.

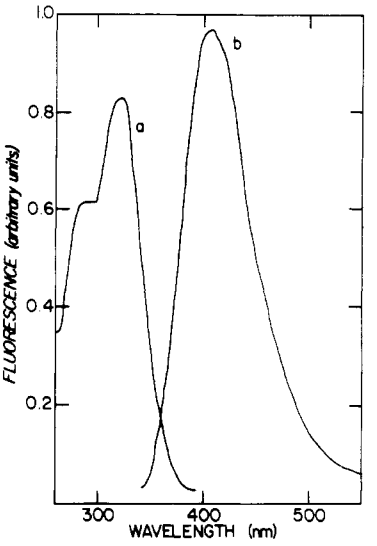


FIGURE 10: Corrected fluorescence excitation and emission spectra of the cofactor–peptide of methylamine dehydrogenase after reduction with dithionite. 3.3 nmol of peptide in 3.0 mL of 50 mM sodium pyrophosphate, pH 7.7, was made anaerobic as described under Materials and Methods. To this solution was added 5.2 nmol of sodium dithionite. The emission wavelength for excitation spectrum a was 410 nm, and the excitation wavelength for emission spectrum b was 320 nm.

at this point we are unable to say what contribution the reduced cofactor itself has on the spectral properties at 280 nm of the cofactor peptide.

Discussion

Physical Properties of Methylamine Dehydrogenase from Bacterium W3A1. Methylamine dehydrogenase, from the organism used in this study, has a molecular weight of 127 000 and is composed of two polypeptides of M_r 45 000 and 15 500. The cofactor of the enzyme absorbs light in the visible region and is covalently linked to the lower molecular weight subunit. Table III presents data on several physical properties of bacterial methylamine dehydrogenases thus far characterized. All these proteins have an A_2B_2 type subunit composition, similar A_{278nm}/A_{429nm} absorbance ratios, and nearly identical absorbance spectra (Figure 1; Eady & Large, 1968; Shirai et al., 1978; Matsumoto, 1978). The cofactor is linked covalently to the lower molecular weight subunit in the enzyme from each of these sources.

Table III: Comparison of Molecular Weight and Spectral Properties of Methylamine Dehydrogenase from Various Sources

source of enzyme	molecular weight: native enzyme	large subunit	small subunit	A_{278nm}/A_{429nm}
bacterium W3A1[a]	127 000	45 000	15 500	7.41
Ps. AM1	105 000[b]	40 000[b]	13 000[b]	7.32[c]
	133 000[c]			
Ps. sp J	105 000[d]	40 000[d]	13 000[d]	8.05[e]

[a] Present study. [b] Shirai et al. (1978). [c] Eady & Large (1971). [d] Matsumoto & Tobari (1978). [e] Matsumoto (1978).

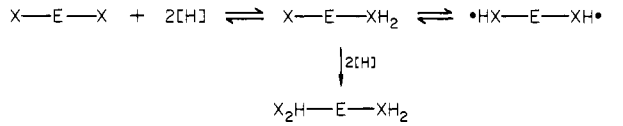
Anaerobic Reductive Titrations of Methylamine Dehydrogenase. Some of the more striking features of this enzyme are the absorbance changes which occur on reduction of the enzyme. Similar spectral changes are seen whether the anaerobic titration is performed at pH 9.0 or at pH 7.7 when either sodium dithionite or methylamine are used as the reductant. In all situations, there is initially a dramatic increase in absorbance at 429 nm until approximately 2 electron equiv of reductant/mol of enzyme has been added. Subsequent additions of reductant lead to a bleaching of the 429-nm absorbance maximum with concomitant appearance of an absorbance maximum at 331 nm. This latter phase of the titration also requires 2 electron equiv of reductant/mol of enzyme. Under the assumption that there are 2 mol of cofactor/mol of enzyme (cf. Table III) and that each catalyzes a two-electron oxidation of methylamine to formaldehyde and NH_4^+, then 4 electron equiv should be accommodated in methylamine dehydrogenase. From the titration shown in Figure 2, 4 electron equiv/mol of enzyme was required to complete the absorbance changes, in very good agreement with the postulated stoichiometry of 2 mol of cofactor/mol of enzyme. Comparable stoichiometrics are observed in the reductive titrations of an enzyme–NH_4^+ complex (Figure 4).

The reductive titration experiments (cf. Figures 2 and 4) indicate collectively that the first and second phases each involves a two-electron reduction of the enzyme. In about one-third of the experiments, however, the first phase in the titration consumed somewhat less of the reductant (dithionite or methylamine) than did the second phase, whether or not NH_4^+ was present during the reductive titrations. The reasons for these variations are unclear at present, but may be ascribed, in part, to the possibility of not having reached an end point for each aliquot of reductant added because of the slow nature of the reduction, and, to a lesser extent, to the enzyme not being fully oxidized at the beginning of the titration.

Since methylamine dehydrogenase can accept 4 electron equiv/mol of enzyme, it is important to consider the oxidation level of the enzyme at the intermediate stage of reductive titration (at the stage represented by curve 2 in Figure 2A). If the reductant were simply titrating each of the two cofactors of the enzyme molecule, a single phase for change in absorbance vs. added reductant would be expected. The fact that biphasic absorbance changes are seen (Figure 2) indicates that the reduction is somewhat more complex. The obvious possibility is that at the intermediate stages in titration, i.e., after the addition of 2 electron equiv, each of the cofactors contains one electron and is thus in a radical form. Evidence for this interpretation is seen in the ESR spectra shown in Figure 5. In the absence of NH_4^+, the spectral changes associated with the first phase of the reductive titration occur faster than those of the second phase, and the intermediate species represented by curve 2 in Figure 2A, i.e., a radical form, can thus be generated by adding excess sodium dithionite to an anaerobic solution of the enzyme. The signal obtained (lower spectrum of Figure 5) decreased slowly, in line with the slow reaction of dithionite in the second reductive phase (see legend to Figure 2). The peak to peak width was 7.5 G, and the g value was 2.0086, larger than that reported for methylamine dehydrogenase from *Ps.* AM1 [g = 2.0038 (De Beer et al., 1980)] and *Ps.* sp J [g = 2.002 (Matsumoto & Tobari, 1978)]. As isolated, the oxidized enzyme from bacterium W3A1 has ca. 5% of the radical compared to the species represented in Figure 5 (lower spectrum). Since the absorption spectra of the enzyme from all three sources is very similar (cf. Table III), we suspect that the radical studied by these other two laboratories represents only a small fraction of that potentially attainable.

In the presence of NH_4^+, the enzyme radical form can be prepared by adding excess dithionite and then allowing the enzyme to partially reoxidize. Since reoxidation of the second reductive step (Figure 4B) occurs much more rapidly than the first phase, the enzyme species represented by curve 2 of Figure 4A is present for a sufficient time for ESR analysis. From a comparison of the ESR and absorption spectra obtained on reductive titration, it is thus possible to conclude that the enzyme species represented by curves 2 in Figures 2A and 4A are radical forms of the enzyme.

Initially, a two-electron step would be expected in the oxidation of methylamine by its dehydrogenase. Comparison of the absorption spectra at the intermediate stage of methylamine titration with that of dithionite reduction indicates that a radical intermediate is also formed with the substrate. The simplest explanation for formation of the radical during the titration with methylamine involves a rapid two-electron reduction of one of the cofactors of the enzyme, followed by a comproportionation between this reduced cofactor and the second oxidized cofactor to the radical state, which is accompanied by the large change in absorbance at 429 nm, i.e.



where E is the enzyme, X is the cofactor, and H represents the added reducing equivalents. The radical ·HX–E–XH·, is quite stable relative to the oxidized and reduced form of the cofactors in X–E–XH_2. The slower reaction of the second phase (several times slower than the first) indicates that the equilibrium of the above reaction mechanism lies far to the right, in favor of ·HX–E–XH·, and that very little oxidized cofactor, X–E–XH_2, is present to react with additional reducing equivalents. Whereas the radical species can react with oxygen to regenerate oxidized enzyme, the reduced form of the enzyme, H_2X–E–XH_2, is essentially inert. A qualitatively different picture emerges when NH_4^+ is included during the titration in that the fully reduced enzyme reoxidizes more rapidly than the radical form.

Interaction of NH_4^+ with Methylamine Dehydrogenase. Several lines of evidence indicate that NH_4^+ forms a specific complex with methylamine dehydrogenase. These include differences in rates of anaerobic reduction with methylamine or dithionite, differences in line width of the ESR spectra of the radical species (Figure 5) relative to the NH_4^+-free enzyme, and differences in the rate of reoxidation of fully reduced or half-reduced enzyme species. A K_D value for dissociation of the NH_4^+–enzyme complex of 15.4 mM at pH 7.7 is obtained from spectral changes observed on titration of the enzyme with ammonium sulfate (Figure 3), and a value of 16.6

mM for this constant is obtained from steady-state kinetic analyses (Table I). The NH_4^+ complex represented by this K_D value may be similar to those which form on binding of alkylamines prior to oxidation.

Inclusion of NH_4^+ in steady-state assays of methylamine dehydrogenase increases the V_{max} and the K_m for methylamine. This suggests that the enzyme has a regulatory site for NH_4^+. One possibility is that NH_4^+ reacts at a carbonyl center of the cofactor. This iminoquinone complex could then be responsible for the increased rate, relative to the quinone form. Another possibility would involve the imine complex at only one of the two cofactor moieties on the enzyme. The modified cofactor then exerts an effect on the interaction of the other cofactor with substrate.[3] The results of the semicarbazide, hydroxylamine, and hydrazine titrations, i.e., 1 mol of titrant/mol of enzyme, offer evidence for possible inequality of binding sites.

Another explanation for the increased V_{max} in the presence of NH_4^+ may be due to a faster rate-limiting reoxidation of the enzyme by PMS. The site of electron transfer to PMS or O_2 may be more exposed or more reactive. The more rapid reoxidation of the fully reduced enzyme by O_2 when NH_4^+ is included supports this proposition. The first-order inactivation of the enzyme in the presence of NH_4^+ during turnover (Table II) also supports the notion of a more exposed site, in that certain group(s) may be more susceptible to modification by PMS. The irreversible inhibition of the enzyme during PMS/DCIP assays when NH_4^+ is present offers evidence for a second, nonsubstrate binding site for this cation, because the saturating concentration of methylamine does not protect the enzyme from inhibition, and increases in NH_4^+ concentration produces a more rapid inactivation.

NH_4^+ is an activator for the oxidation of alcohols by the quinoprotein methanol dehydrogenase (Anthony & Zatman, 1967; Duine et al., 1978). Interestingly, if NH_4^+ is incubated with the corresponding enzyme from bacterium W3A1 in the presence of PMS and DCIP, but in the absence of alcohol, enzyme-catalyzed dye reduction occurs, and the rate of reaction goes to zero following pseudo-first-order kinetics. Activity cannot be recovered by the addition of alcohol.[2] When both methanol and NH_4^+ are present at the start of the enzymic reaction, then this inhibition is not observed. Clearly, NH_4^+ is a potent activity modulator of quinoproteins.

Interaction of Methylamine Dehydrogenase with Formaldehyde. Formaldehyde, the second product of methylamine oxidation by methylamine dehydrogenase, also has some effect on the enzyme. A mixture of NH_4^+, formaldehyde, and oxidized enzyme produces a reduced type enzyme spectrum (Figure 6). Possibly it is the imine, $H_2C{=}NH_2^+$, the Schiff base of NH_4^+ and formaldehyde, which causes the spectral perturbations, since the spectrum of the NH_4^+-free enzyme is unaltered by added formaldehyde.

Effect of 2-Bromoethylamine, Semicarbazide, Hydroxylamine, and Hydrazine on Methylamine Dehydrogenase. Incubation of the enzyme with 2-bromoethylamine led initially to an enzyme form with spectral properties similar to those of fully reduced enzyme (cf. curve 2 of Figure 2B). On standing, the spectral properties slowly changed to that of the intermediate, i.e., radical, species (cf. curve 1 of Figure 2B), indicative of comproportionation of the 2-bromoethylamine-derived electrons. The enzyme thus obtained was inactive. These experiments suggest irreversible modification of the enzyme occurs in a single—or, at most, a few—turnover(s). Oxidative deamination of this compound produces α-bromoacetaldehyde which can react with several types of functional groups of enzymes, especially sulfhydryl groups.

The reaction of semicarbazide, hydroxylamine, and hydrazine with methylamine dehydrogenase was not unexpected from the quinone structure of the cofactor (Finley, 1974). Whereas titrations with methylamine or dithionite require 2 mol of reductant/mol of enzyme, i.e., 1 mol/mol of cofactor, only 0.5 mol/mol of cofactor (Figures 7 and 8) on the enzyme appears to be altered by semicarbazide, hydrazine, or hydroxylamine. This would suggest half-the-sites reactivity for methylamine dehydrogenase. If one site is occupied by semicarbazide, hydroxylamine, or hydrazine (or, presumably, NH_4^+ or methylamine), the other site cannot react or has altered reactivity. The essential irreversibility of semicarbazide, hydroxylamine, and hydrazine binding would eliminate any oxidation of substrate. Half-the-site reactivity is also suggested by the experiment in which 2-bromoethylamine is added to methylamine dehydrogenase. The enzyme undergoes a two-electron reduction, i.e., one of two cofactors per mol of enzyme, followed by comproportionation into two radicals presumably on the same enzyme molecule.

Nature of the Cofactor of Methylamine Dehydrogenase from Bacterium W3A1. The cofactor peptide isolated from methylamine dehydrogenase requires 2 electron equiv/mol to bleach the absorbance at 424 nm (Figure 9). This observation further supports our hypothesis that each cofactor within the enzyme has a two-electron capacity. Comparison of Figure 9 with Figure 2 suggests that the quaternary structure is required for stabilization of the radical form of the native enzyme (Figure 5).

De Beer et al. (1980) have suggested that the cofactor of methylamine dehydrogenase from *Ps.* AM1 is methoxatin (2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione) covalently linked to the protein. This conclusion is based primarily upon ESR and ENDOR spectra of the radical form of the enzyme. The visible and fluorescent spectral properties of oxidized, radical, and reduced forms of methylamine dehydrogenase and its cofactor peptide reported in this paper are substantially different from those of methoxatin and several of its adducts (Dekker et al., 1982) and from those of enzymes containing this cofactor. Although the ESR data (cf. above; De Beer et al., 1980) suggest a quinone nature to the cofactor, further studies are necessary to ascertain its relationship to methoxatin. It is highly likely, nevertheless, that the cofactors of the various methylamine dehydrogenases thus far examined are very similar, if not identical.

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Registry No. NH_4^+, 14798-03-9; methylamine, 74-89-5; methylamine dehydrogenase, 60496-14-2; 2-bromoethylamine, 107-09-5; semicarbazide, 57-56-7; hydrazine, 302-01-2; hydroxylamine, 7803-49-8; formaldehyde, 50-00-0.

References

Anthony, C., & Zatman, L. J. (1967) *Biochem. J. 104*, 953–959.

Benesi, H. A., & Hildebrand, J. H. (1949) *J. Am. Chem. Soc. 71*, 2703.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177–2185.

Corey, E. J., & Tramontano, A. (1981) *J. Am. Chem. Soc. 103*, 5599–5600.

[3] For a discussion of this model, see Segel (1975).

De Beer, R., Duine, J. A., Frank, J., Jzn., & Large, P. J. (1980) *Biochim. Biophys. Acta 622*, 370-374.
Dekker, R. H., Duine, J. A., Frank, J., Jzn., Verwiel, P. E. J., & Westerling, J. (1982) *Eur. J. Biochem. 125*, 69-73.
Duine, J. A., & Frank, J. Jzn. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 278-280.
Duine, J. A., Frank, J., & Westerling, J. (1978) *Biochim. Biophys. Acta 524*, 277-287.
Duine, J. A., Frank, J., Jzn., & Verwiel, P. E. J. (1980) *Eur. J. Biochem. 108*, 187-192.
Eady, R. J., & Large, P. J. (1968) *Biochem. J. 106*, 245-255.
Eady, R. J., & Large, P. J. (1971) *Biochem. J. 123*, 757-771.
Edmondson, D. E., & Singer, T. P. (1973) *J. Biol. Chem. 248*, 8144-8149.
Edmondson, D. E., & Singer, T. P. (1976) *FEBS Lett. 64*, 255-265.
Finley, K. T. (1974) in *The Chemistry of Quinone Compounds* (Patai, S., Ed.) Part 2, pp 877-1144, Wiley, London.
Fox, T., Hinkley, D., & Larntz, K. (1980) *Technometrics 22*, 29-33.
Gainor, J. A., & Weinreb, S. M. (1982) *J. Org. Chem. 47*, 2833-2837.
Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 77*, 751-766.
Kenney, W. C. (1980) in *Flavins and Flavoproteins* (Yagi, K., & Yamano, T., Eds.) pp 237-243, Japan Scientific Societies Press, Tokyo.
Matsumoto, T. (1978) *Biochim. Biophys. Acta 522*, 291-302.
Matsumoto, T., & Tobari, J. (1978) *J. Biochem. (Tokyo) 83*, 1591-1597.
Oppenheimer, L., Capizzi, T. P., & Miwa, G. F. (1981) *Biochem. J. 197*, 721-729.
Ralston, M. L., & Jennrich, R. I. (1978) *Technometrics 20*, 7-14.
Salisbury, S. A., Forrest, H. S., Cruse, W. B. T., & Kennard, O. (1979) *Nature (London) 280*, 843-844.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 387-388, Wiley, New York.
Shirai, S., Matsumoto, T., & Tobari, J. (1978) *J. Biochem. (Tokyo) 83*, 1599-1607.
Singer, T. P., & Kearney, E. B. (1950) *Arch. Biochem. Biophys. 29*, 190.
Singer, T. P., & Kenney, W. C. (1974) *Vitam. Horm. (N.Y.) 32*, 1-45.
Steenkamp, D. J., & Mallinson, J. (1976) *Biochim. Biophys. Acta 429*, 705-719.
Steenkamp, D. J., McIntire, W., & Kenney, W. C. (1978) *J. Biol. Chem. 253*, 2818-2824.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048-4052.
Suva, R. H., & Abeles, R. H. (1978) *Biochemistry 17*, 3538-3545.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.

Changes in Histone H3 Composition and Synthesis Pattern during Lymphocyte Activation†

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ABSTRACT: Freshly isolated human lymphocytes were found to synthesize histones at a significant rate even though no DNA was being synthesized. The synthesis pattern of histone variants in resting lymphocytes is similar to that found in other quiescent cells and different from that found in S-phase cells. For this reason, the histone synthesis in resting lymphocytes cannot be attributed to contamination by S-phase cells. Stimulation by the mitogen phytohemagglutinin resulted in a dramatic switch in the histone H3 variant synthesis pattern as well as a readily apparent change in the histone H3 mass pattern. Thus, the chromatin of activated lymphocytes has a different histone H3 variant composition than resting or quiescent lymphocytes. It is suggested that the proportion of H3.3 in the mass pattern of the chromatin of a cell may be related solely to how long that cell has been quiescent. Inducing resting lymphocytes to synthesize DNA by UV irradiation did not qualitatively change the histone variant synthesis pattern. No S-phase H3 variants were induced by the repair process. Furthermore, the quantity of histone synthesized neither increased nor decreased after treatment with UV light.

Recently, this laboratory (Wu & Bonner, 1981; Wu et al., 1982b) has shown that not all histone synthesis occurs during DNA replication (S phase) but that a significant amount occurs during the G_1 and G_2 phases of the cell cycle and during the quiescent or G_0 state. The variant patterns of the histone synthesis in quiescent, S-phase, and G_1/G_2-phase cells all differed from each other. Specifically, H3.3 was the only H3 variant synthesized in quiescent and in G_1/G_2 cells. All four H2A variants were synthesized in quiescent and S-phase cells, but only H2A.X and H2A.Z were synthesized in G_1/G_2 cells. No part of the G_1 or G_1/S transition could be found with a H2A synthesis pattern like that in quiescent cells (Wu et al., 1982b).

These results have implications in two quite different areas. The first is that histone synthesis is not necessarily linked to DNA synthesis. The second is that the histone variant synthesis pattern may usefully serve as an indicator of the physiological state of the cell. Since these observations and conclusions were drawn from studies performed on continuous

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