N-Methylglutamate Synthetase

SUBSTRATE-FLAVIN HYDROGEN TRANSFER REACTIONS PROBED WITH DEAZAFLAVIN MONONUCLEOTIDE*

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SUMMARY

N-Methylglutamate synthetase, reconstituted from apoprotein with 5-deazaFMN, catalyzes the reversible formation of N-methylglutamate via the same two-step mechanism previously elucidated for native enzyme (Reactions 1 and 2).

\[
\text{deazaFMNH}_2
\]

\[
E\text{-deazaFMN} + L\text{-glutamate} \rightleftharpoons E + \text{ammonia} \quad (1)
\]

\[
\text{glutaryl}
\]

\[
\text{deazaFMNH}_2
\]

\[
E + \text{methylamine} \rightleftharpoons E\text{-deazaFMN} \quad (2)
\]

\[
\text{glutaryl}
\]

+ N-methyl-L-glutamate

This conclusion is based on the observation that:
1. Enzyme-bound deazaFMN (\(\lambda_{\text{max}} = 410, 338, \epsilon_{490} = 10,400 \text{ M}^{-1} \text{ cm}^{-1}\)) is reduced by L-glutamate, N-methyl-L-glutamate but not L-glutamate. At saturating concentrations of L-glutamate Reaction 1 proceeds at 1% of the rate observed with FMN-reconstituted enzyme.

2. Substrate-reduced deazaFMN enzyme is reoxidized by methylamine or ammonia.

3. A glutaryl enzyme intermediate, isolated by Sephadex G-25 chromatography, contains radioactivity when prepared from [U-\(^{14}\text{C}\)]glutamate, [\(\alpha\)-\(^{3}\text{H}\)]glutamate, or N-[\(\alpha\)-glutaryl-\(^{14}\text{C}\)]methylglutamate; however, this intermediate is not labeled from N-[methyl-\(^{14}\text{C}\)]methylglutamate.

4. The amount of radioactivity incorporated into the intermediate is stoichiometric with the amount of deazaFMN reduced during its formation.

5. Intermediate prepared with [U-\(^{14}\text{C}\)]glutamate yields \(\alpha\)-[\(^{14}\text{C}\)]ketoglutarate when denatured with acid and N-[\(\alpha\)-glutaryl-\(^{14}\text{C}\)]methylglutamate when incubated with methylamine. In the absence of methylamine deazaFMN enzyme intermediate slowly decays to yield \(\alpha\)-hydroxyglutarate.

6. The rate of deazaFMN glutaryl enzyme intermediate formation at a fixed glutamate concentration is equal to the rate of the over-all reaction while the rate of intermediate reaction with methylamine is approximately 50 times greater than the over-all reaction.

DeazaFMN enzyme intermediate prepared with [\(\alpha\)-\(^{3}\text{H}\)]glutamate yields [\(^{3}\text{H}\)]deazaFMNH\(_2\) when denatured with acid or phenol and N-[\(\alpha\)-\(^{3}\text{H}\)]methylglutamate when incubated with methylamine. These results show that the \(\alpha\)-hydrogen of glutamate is transferred to deazaFMNH\(_2\), presumably at the 5 position, during Reaction 1 and that the same hydrogen is utilized for the reformation of the \(\alpha\) C—H bond during Reaction 2. These results provide the first direct evidence for enzymic hydrogen transfer from substrate to flavin.

The flavoenzyme, N-methylglutamate synthetase (1, 2) catalyzes the reversible formation of N-methyl-L-glutamate from L-glutamate and methylamine via the two-step mechanism shown in Reactions 1 and 2 (3).

\[
\text{FMN reduced}
\]

\[
E\text{-FMN} + L\text{-glutamate} \rightleftharpoons E + \text{ammonia} \quad (1)
\]

\[
\text{glutaryl}
\]

\[
\text{FMN reduced}
\]

\[
E + \text{methylamine} \rightleftharpoons E\text{-FMN} \quad (2)
\]

\[
\text{glutaryl}
\]

+ N-methyl-L-glutamate

It has been shown that the \(\alpha\)-hydrogen abstracted from glutamate during formation of the reduced enzyme intermediate (Reaction 1) is not exchangeable with solvent, and is used in the reformation of the \(\alpha\) carbon-hydrogen bond, (Reaction 2) (3). The \(\alpha\) substrate hydrogen may be transferred to a basic amino acid residue in the protein or to the flavin prosthetic group during formation of the reduced enzyme intermediate.

Hydrogen attached to the weakly basic nitrogens in free reduced flavins exchanges rapidly with solvent, preventing direct study of substrate-flavin hydrogen transfer during catalysis. This difficulty might be avoided by use of the flavin analogue deaza-
FMN in which the N-5 nitrogen of FMN is replaced by CH (4, 5). Flavins and deazaflavins have been shown to exhibit many similar properties: (a) Both react with sulfite to form a covalent adduct at the 5 position (6, 7); (b) both are chemically reduced by pyridine nucleotides (7) or by dithionite (5) and photochemically reduced in the presence of EDTA (5); (c) reduced flavins and reduced deazaflavins can be reoxidized by oxygen (5), cytochrome c (5), carbonyl compounds (8) and disulfides (7); (d) both compounds in the oxidized state form nonfluorescent complexes with tryptophan and β-resorcylic acid (7); and (e) theoretical calculations indicate that the 5 position is the most electrophilic position (5), deazaflavins are more slowly reduced than flavin and the reduced species is more slowly reoxidized as compared with tryptophan and β-resorcylic acid (7); and (e) theoretical calculations indicate that the 5 position is the most electrophilic position (5), deazaflavins are more slowly reduced than flavin and the reduced species is more slowly reoxidized as compared with tryptophan and β-resorcylic acid (7). The results of this study are the subject of this paper. A preliminary communication describing these results has previously been published (10).

**EXPERIMENTAL PROCEDURE**

**Materials**

Deazariboflavin was synthesized according to the method of O'Brien et al. (11). This compound was phosphorylated according to the procedure of Flexser and Farkas (11). DeazaFMN and commercial FMN (Sigma Chemical Co.) were purified in the dark on DEAE-cellulose as described by Massey and Swoboda (12). DeazaFMN was judged pure since it showed a single blue fluorescent spot in paper (Whatman No. 3MM) chromatography (5% NaHPO/0.2HCl in water; l-butyl alcohol:6H2O:water, 80:2:20) and thin layer chromatography (ethyl acetate-formic acid-water (7:2:1)). The preparation of N-[l-glutaryl-L-4C] and N-[methyl-4C]methylglutamate was performed as described by Pollock and Hersh (3). [14C]Methylamine hydrochloride and L-[U-14C]Glutamate was obtained from ICN. N-Methyl-t-glutamic acid at 0°. During a period of 20 s, 2.0 ml of 3.0 M potassium phosphate buffer, pH 7.4, containing 50 mM L-glutamate, 5 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 1 mM magnesium disodium EDTA, was added. After an additional 40 s, during which mixing was continued, an additional 8.0 ml of the ammonium sulfate solution were added. The precipitated protein was collected by centrifugation, redissolved in 2.0 ml of 100 mM potassium N-tris(hydroxymethyl)methylglycine (Tricine) buffer, pH 9.1, containing 30 mM L-glutamate, 5 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 1.5 mM potassium bromide, and stored at 0° for 30 min. The ammonium sulfate precipitation procedure was repeated two additional times, except that the addition of the 3.0 M potassium bromide solution was omitted, and the final precipitate was dissolved in 2.0 ml of 100 mM potassium Tricine buffer, pH 9.1, containing 50 mM L-glutamate, 5 mM magnesium chloride, 5 mM 2-mercaptoethanol, and 1 mM magnesium disodium EDTA. This solution was dialyzed for 4 hours versus 2.5 liters of the same potassium phosphate buffer, pH 7.4, containing 100 mM potassium phosphate, 5 mM 2-mercaptoethanol, 1 mM magnesium disodium EDTA, and 5 mM magnesium chloride. The apoprotein solution (20 mg/ml) was centrifuged after dialysis to remove small amounts of denatured protein and reconstituted with FMN or deazaFMN by incubating with 0.15 M flavin at 13° for 30 to 60 min. Reconstituted enzyme was dialyzed for 14 hours versus 250 ml of the same buffer using dialysis of ammonium sulfate, and stored at -70°. Unless otherwise indicated, this buffer was used in experiments with apoprotein and reconstituted enzyme.

**Spectral Determinations**—Absorption spectra were obtained using a Cary 14 spectrophotometer equipped with a thermostatted cell chamber maintained at 8° to 10°. A specially constructed cuvette, equipped with two side arms and a ground glass stopcock, was used for spectra recorded under anaerobic conditions. Samples were made anaerobic by flushing the cuvette with argon (99% argon containing 1% hydrogen) from which traces of oxygen were removed by passing the gas stream through a Decox gas purifier (Matheson model 64-1000). Measurements involving changes in absorbance at a single wavelength were done either with a Cary 14 or Gilford spectrophotometer.

Extinction coefficients were determined by two methods: (a) A sealed tube containing enzyme was heated for 5 min in a boiling water bath in the dark. After cooling, the precipitated protein was removed by centrifugation, and the flavin content of the supernatant was determined by its spectrum. (b) The spectrum of the enzyme was recorded before and after the addition of 0.9 M urea and 0.5 M sodium thiocyanate. The difference spectrum results in the release of flavin from enzyme (as determined from the differences in 0.5 and fluorescent changes between bound and free flavin) without protein precipitation. Neither procedure causes spectral changes with free FMN or deazaFMN and results from both methods agree within ±2%. Calculations were made using a molar extinction coefficient of 12.5 x 104 cm⁻¹ M⁻¹ for free FMN (11) and a value of 12.0 x 104 cm⁻¹ M⁻¹ for free deazaFMN (4).

The rate of reduction of bound flavin in FMN- and deazaFMN-reconstituted enzyme was determined by monitoring the decrease in absorbance at 450 and 410 nm, respectively, after addition of L-glutamate. The reactions were performed at 8° in 125 mM potassium Tricine buffer, pH 8.3, containing 1.25 mM dithioerythritol, 12.5 mM potassium disodium EDTA, and 0.4 M potassium magnesium disodium EDTA. The pseudo-first order rate constants for each reaction were determined by plotting the data according to the method of Guggenheim (16).

The amounts of methylamine and α-ketoglutarate produced during reaction of deazaFMN enzyme with N-methylglutamate were determined after denaturation of the enzyme with trichloroacetic acid. All determinations were performed at room temperature using a 0.5 M succinate buffer, pH 7.4, 7 x 10⁻⁴ M 2,6-dichlorophenolindophenol, sample, and water to 0.5 ml. The error in these determinations was found to be ±0.2 mmol. α-Ketoglutarate was determined using glutamate dehydrogenase, according to the method of Bergmeyer and Bernt (18). Each assay mixture contained 0.5 mM potassium phosphate, pH 7.4, 3.0 M ammonium sulfate, 1.5 mM EDTA, 25 mM ammonium sulfate, 0.128 mM NADH, sample, and water to 0.5 ml. The error in these measurements was found to be ±1.0 mmol.

**Product Identification—Samples containing glutamate or N-methylglutamate were chromatographed on DEAE-cellulose (Whatman No. 3MM) and thin layer (Silica Gel F-254) chromatography, as previously described (10). Radioactivity was located by autoradiography.
glutamate dehydrogenase, under conditions similar to those described by Williamson and Corkey (20). The amount of glutamate thus formed was determined by chromatography on a Dowex 50 column.

Prior to identification of α-hydroxyglutarate, enzyme intermediate was denatured with 5% trichloroacetic acid and radioactive α-ketoglutarate, if present, was removed by formation and extraction of its 2,4-dinitrophenylhydrazone. The sample was then applied to a Dowex 50 column and α-hydroxyglutarate was eluted by washing with water (residual 2,4-dinitrophenylhydrazone and deazaFMN remained on the column). Trichloroacetic acid was removed by ether extraction, carrier α-hydroxyglutarate was added, and the sample was concentrated by evaporation with ethanol at 40° under a stream of air. After chromatography on Whatman No. 3MM paper in the following solvent systems: 1-butanol-acetic acid-water (12:3:5), isopropyl ether-formic acid-water (4:1:1), α-hydroxyglutarate was visualized by spraying with bromphenol blue (150 mg in 250 ml of ethanol containing 0.19 ml of 5 N sodium hydroxide). Radioactivity was located by radioautography prior to use of the spray reagent. Alternatively, the chromatogram (30 to 35 cm from origin to solvent front) was cut into 5-mm strips, α-hydroxyglutarate was eluted by shaking in 1.0 ml of water for 24 hours, and the radioactive content of each fraction was determined by liquid scintillation counting. The location of the radioactive peak was compared to parallel strips, run between and on either side of the unknowns, on which the α-hydroxyglutarate was located by the bromphenol blue spray reagent.

α-[3H]Hydroxyglutarate was also identified following reaction of deazaFMN enzyme with α-[3H]glutamate without prior isolation of the enzyme intermediate. Enzyme was reacted with α-[3H]glutamate under the same conditions used for the preparation of enzyme intermediate except commercial α-[3H]glutamate was purified before use by chromatography on Dowex 50 to remove small amounts of impurities which elute from Dowex in water. The enzyme was then denatured with 5% trichloroacetic acid and chromatographed on Dowex 50 as described above. The amount of α-hydroxyglutarate formed was determined from the amount of nonvolatile counts which eluted from Dowex 50. α-Hydroxyglutarate was identified by paper chromatography as described above and radioactivity was located by fluorography (21).

Tritium labeled deazaFMNH₂ was released from enzyme intermediate prepared with α-[3H]glutamate by two methods. (a) The intermediate was denatured with 0.25 M formic acid. (b) DeazaFMNH₂ was extracted from the intermediate (0.7 ml) with phenol (0.7 g). The phenol phase was then diluted with 0.4 ml of water and the phenol was removed by ether extraction whereas deazaFMNH₂ remained in the aqueous phase. Tritium-labeled deazaFMN was identified in each case after air oxidation.

RESULTS

Reconstitution of Apoprotein with FMN and DeazaFMN—The addition of either FMN or deazaFMN to the apoprotein of N-methylglutamate synthetase results in the binding of the chromophores, as indicated by the quenching of fluorescence of the free flavin derivatives. In the presence of excess apoprotein the reaction follows pseudo-first order kinetics and similar rate constants are obtained for the binding of FMN (kₜₐ = 3.3 min⁻¹) and deazaFMN (kₜₐ = 2.9 min⁻¹) (Fig. 1). A bathochromic shift of the longer wavelength band of FMN (445 nm) and deazaFMN (397 nm) occurs upon binding to apoprotein. The absorption spectrum of FMN-reconstituted enzyme shows absorption maxima at 370 and 452 nm and a minimum at 398 nm, identical with those of native enzyme. Enzyme-bound deazaFMN exhibits absorption maxima at 338 and 410 nm and a minimum at 362 nm (Fig. 2). The molar extinction coefficients of FMN enzyme at 450 nm and of deazaFMN enzyme at 410 nm are 11,400 and 10,400, respectively. The ratios, milligrams of protein to A₄₅₀ and milligrams of protein to A₄₄₅, have been used to estimate the extent of reconstitution with FMN and deazaFMN, respectively. This ratio varies between 11.0 and 12.5 for native enzyme preparations. Preparations of enzyme reconstituted with either FMN or deazaFMN yield values within the range 18.0 to 24.0, indicating 50 to 70% reconstitution.

The catalytic efficiency of deazaFMN-reconstituted enzyme as compared to FMN-reconstituted enzyme is shown in Fig. 3. That the deazaFMN enzyme is catalytically active is evidenced by the fact that 100 turnovers per mol of enzyme-bound deazaFMN have occurred at the 120-min time point. The FMN-reconstituted enzyme exhibits a specific activity equal to 69% of the native enzyme while the specific activity of the deazaFMN enzyme is 3.3% of the native enzyme.

Reduction and Reoxidation of DeazaFMN Enzyme by Substrates—Flavin reduction occurs during the reaction of native enzyme or FMN-reconstituted enzyme with amino acid substrates, according to Reaction 1. A similar reaction is observed with deazaFMN

![Fig. 1](http://www.jbc.org/)
Fig. 3. Time course of N-methylglutamate formation from glutamate and methylamine in the presence of FMN-reconstituted (1) and deazaFMN-reconstituted (2) enzymes and apoprotein (3). Line 1 is plotted according to the scale on the right and Lines 2 and 3 are plotted according to the scale on the left. Enzyme activity was measured at 30° by the radioisotope assay previously described (2). Specific activity values (micromoles of N-methylglutamate/min/mg of protein $\times 10^3$) calculated for native, FMN-reconstituted, and deazaFMN-reconstituted enzymes are 115.0, 79.4, and 3.8, respectively. The latter two values are corrected for the blank reaction observed with apoprotein alone.

Fig. 4. Reduction of deazaFMN enzyme by L-glutamate. Curve 1 is the spectrum of oxidized enzyme in 110 mM potassium Tricine buffer, pH 8.3, containing 16.7 mM potassium chloride, 1.1 mM dithioerythritol, 1.4 mM magnesium chloride, and 0.3 mM magnesium disodium EDTA. Curves 2, 3, and 4 were recorded 1.5, 11.5, and 34 min, respectively, after the addition of 22.2 mM L-glutamate at 8°.

Fig. 5. Anaerobic dithionite titration of deazariboflavin in 100 mM potassium Tricine buffer, pH 8.5, at 8°. Curve 1 is oxidized deazariboflavin (13.2 $\mu$M) and Curves 2 and 3 were recorded 40 min after the addition of 5.3 and 8.5 $\mu$M dithionite, respectively.

deazaFMN/mol of enzyme (native enzyme contains 2 mol of flavin/mol of enzyme (3)). FMN enzyme was added to this preparation such that the concentration of residual FMN enzyme was increased 2-fold in one experiment and 5-fold in a separate experiment. The half-times for reduction of deazaFMN by 25 mM glutamate at 10° were 3.9 min for the original deazaFMN preparation, and 4.0 and 3.8 min for the 2-fold and 5-fold increase in FMN enzyme, respectively. At 410 nm, the wavelength at which deazaFMN reduction is measured, no appreciable spectral changes are observed for reduction of FMN (3). Thus, if FMN enzyme was catalyzing the reduction of deazaFMN enzyme, the measured half-times would have had to decrease 2-fold and 5-fold respectively. We, therefore, conclude that deazaFMN enzyme reacts directly with glutamate, and thus is, in itself, enzymatically active.

Substrate reduction of deazaFMN enzyme leads to the appearance of a new absorption band at 325 nm and an isosbestic point at 334 nm. This latter observation is consistent with the direct conversion of oxidized deazaFMN to a reduced species without the participation of any spectrally discernible intermediate. The spectrum of the reduced species is consistent with the formation of 1,5-dihydrodeazaFMN since a similar hypochromic shift of the 340 nm band of the oxidized chromophore also occurs in the case of free deazariboflavin during titration with dithionite and partially reduced samples of deazariboflavin show an absorption maximum at 320 nm (Fig. 5). A similar absorption maximum was not observed by Edmondson et al. (5) during the photoreduction of deazariboflavin in the presence of EDTA. This band may have been obscured in their experiments by the atypically high absorbance of the oxidized chromophore in this region of the spectrum.

In the presence of excess amino acid substrate the reduction of native enzyme (3), FMN-reconstituted enzyme, and deazaFMN-

enzyme upon addition of L-glutamate or N-methyl-L-glutamate (Fig. 4), but not n-glutamate, indicating that in Reaction 1 deazaFMN enzyme exhibits the same specificity as native enzyme.

In order to rule out the possibility that the residual FMN enzyme (see Fig. 3) was acting as a catalyst for the reduction of deazaFMN enzyme, the following experiment was performed. The concentration of FMN enzyme in the deazaFMN enzyme preparation was estimated from Fig. 3 and also from the spectrum of the apoenzyme. A value of 0.079 mol of FMN/mol of enzyme was obtained, while the deazaFMN enzyme contained 1.4 mol of...

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reconstituted enzyme show multiphasic kinetics. There is an initial rapid phase of reduction, amounting to approximately 75% of the total reaction, which follows pseudo-first order kinetics when plotted according to the procedure of Guggenheim (16). The slower phase of flavin reduction is presumed to be due to the presence of partially denatured enzyme since it shows the same isosbestic point as the initial fast phase and is too slow to account for the catalysis observed in turnover experiments. The formation of a single spectrally discernible reduced species is also suggested by experiments with native enzyme which show that the pseudo-first order rate constant for the rapid phase of reduction and the percent of the total reaction occurring in the fast phase is independent of wavelength over the range of 300 to 530 nm. The rates of flavin reduction were therefore calculated from the pseudo first order rate constants obtained during the initial rapid phase of flavin reduction.

Comparison of the rates of glutamate reduction of enzyme-bound FMN and deazaFMN was obtained by measuring the rate of flavin reduction at several glutamate concentrations under conditions described under "Methods" and extrapolating the results to infinite glutamate. With FMN-reconstituted enzyme, plots of 1/kobs versus 1/[glutamate] for 62, 250, 625, and 1250 μM glutamate yielded a value of 20.8 min⁻¹ at infinite glutamate. With deazaFMN enzyme the rate was too slow to measure at low glutamate concentrations. However, a constant value of 0.17 min⁻¹ was obtained at 2.5 and 25 mM glutamate, indicating saturation at the lower concentration. Thus reduction of deazaFMN-reconstituted enzyme proceeds at approximately 1% the rate of FMN-reconstituted enzyme.

Free reduced deazaFMN, released from substrate-reduced enzyme by acid denaturation, is slowly reoxidized (t₁/₂ = 3 hours), similar to results obtained for the reoxidation of 1,5-dihydrdeoazaflavins (5). Rapid reoxidation of enzyme-bound reduced deazaFMN occurs upon addition of either ammonia or methylamine (Fig. 6). Reoxidation in the presence of saturating glutamate with 22.2 mM ammonium sulfate is too fast to measure on the Cary spectrophotometer and at the same concentration of methylamine the reaction is complete within 1 min. These results show that deazaflavin reduction is slower than reoxidation since the t₁/₂ for reduction in the presence of saturating glutamate is 4.0 min.

The addition of a 2-fold excess of ammonia with respect to glutamate causes nearly complete flavin reoxidation, as judged by the increase in absorption at 410 nm (Fig. 6). The extent of reoxidation is difficult to evaluate precisely since the spectrum obtained after reoxidation differs from the original spectrum of the oxidized enzyme, as evidenced most notably by the shift of the absorption maximum from 410 to 415 nm (Fig. 6). Experiments in which the glutamate concentration was held constant and the ammonia concentration varied to give partial flavin reoxidation indicate that the equilibrium strongly favors flavin reoxidation. This can be contrasted with the equilibrium constant of 1 obtained for Reaction 1 with native enzyme (3).

The addition of amine substrate alone to oxidized deazaFMN enzyme does not cause perturbation of the absorption spectrum. However, a perturbation virtually identical to that shown in Curve 3 (Fig. 6), as judged by the position of the new absorption maxima (415, 340 nm) and minimum (370 nm), is observed with nonreducing amino acid substrate analogues, such as d-glutamate, α-ketoglutarate, or L-α-hydroxyglutarate. This suggests that the spectrum obtained after reoxidation with amine substrate is due to an t-glutamate-oxidized enzyme complex. This complex would be expected to be the predominant enzyme species under the conditions of Fig. 6 since the steady state would favor an oxidized form of the enzyme and glutamate is present at saturating levels.

Isolation of a DeazaFMN Glutaryl Enzyme Intermediate.—The reduction-reoxidation reactions observed with deazaFMN enzyme suggested that a deazaFMN glutaryl enzyme intermediate was formed and, as with native enzyme (3), could be isolated by Sephadex G-25 chromatography. Incubation of deazaFMN enzyme with [U-14C]glutamate or [α-3H]glutamate (10) results in the incorporation of radioactivity into the enzyme (Fig. 7). No radioactivity is incorporated when apoprotein is incubated with [U-14C]glutamate. The amount of radioactivity incorporated after reaction with [U-3H] or [α-3H]glutamate is approximately stoichiometric (70 to 90% yields) with the amount of deazaFMN reduced during intermediate formation, indicating that deazaFMN is the sole oxidant of the amino acid substrate. Radioactivity is also incorporated into the enzyme when N-[glutaryl-14C]methylglutamate is used as the oxidant, but virtually no radioactivity chromatographs with enzyme when N-[methyl-14C]-methylglutamate is used (Fig. 7). These results show that the glutaryl group, but not the amino group, of the amino acid substrate is incorporated into the intermediate, indicating that cleavage of the α-carbon-nitrogen bond of the substrate occurs during intermediate formation, similar to native enzyme (3) and consistent with the release of the amino group as the first product of the over-all reaction.

Intermediate prepared with [U-14C]glutamate yields α-[14C]-ketoglutarate when denatured with acid. Reaction of this intermediate with methylamine results in the disappearance of α-ketoglutarate and the formation of stoichiometric amounts of N-methylglutamate (Table I). These results show that the catalytically active glutaryl residue is released as α-ketoglutarate upon acid denaturation of the intermediate and that oxidation at the α carbon of the amino acid substrate occurs during intermediate formation similar to native enzyme (3).

Intermediate prepared from [α-3H]glutamate does not contain radioactive α-ketoglutarate, indicating that intramolecular shift
FIG. 7. Isolation of deazaFMN glutaryl enzyme intermediate.
Enzyme was incubated for 60 min at 10°C in 110 mM Tricine buffer, pH 8.3, 88 mM potassium chloride, 11 mM dithioerythritol, 1 mM magnesium disodium EDTA, 5 mM magnesium chloride, and the indicated substrate at 1 mM concentration and then chromatographed on a Sephadex G-25 column, as previously described (10). Specific activities of the labeled substrates were: [U-14C]glutamate, 3.2 x 10^4 cpm/nmol; N-[glutaryl-U-14C]methylglutamate, 1.2 x 10^4 cpm/nmol; and N-[methyl-14C]methylglutamate, 1.6 x 10^3 cpm/nmol. The amount of labeling observed corresponds to 0.8, 0.5, and 0.08 mol of substrate per mol of reduced deazaFMN formed during reaction with [U-14C]glutamate, N-[glutaryl-U-14C]methylglutamate, and N-[methyl-14C]methylglutamate, respectively. The deazaFMNH2 formed was determined by the decrease in absorbance at 410 nm (e₃₂₅₀ = 9400 M⁻¹ cm⁻¹). Since substrate does not fully reduce deazaFMN enzyme a value for deazaFMNH2 could not be determined and was assumed to be 10% of the value determined for deazaFMN.

| Treatment          | Reaction products of deazaFMN enzyme intermediate formed with [U-14C]glutamate |
|--------------------|----------------------------------------------------------------------------------|
| Acid denaturation  | Products: α-Ketoglutarate 2440 cpm, N-Methylglutamate 0 cpm                        |
| Methylamine        | Products: α-Ketoglutarate 180 cpm, N-Methylglutamate 2230 cpm                    |

TABLE I

of the α hydrogen does not occur. Greater than 70% of the tritium is present in this intermediate as deazaFMNH2. If the intermediate is denatured by acid, 30% of the tritium in deazaFMNH2 is converted to 4H2O during air reoxidation and the remaining 70% of the tritium chromatographs with deazaFMN during paper (Fig. 8) and thin layer chromatography (10). Similar results are obtained if deazaFMNH2 is extracted from the intermediate with phenol as described under "Methods," indicating that the observed incorporation of tritium is not an artifact produced during acid denaturation of the intermediate. If the intermediate is incubated with methylamine prior to acid denaturation, 85% of the tritium, initially present as deazaFMNH2, is incorporated into N-methylglutamate (10). Intermediate prepared from native enzyme or FMN-reconstituted enzyme with [α-3H]glutamate yields 4H2O when denatured with acid and N-[3H]methylglutamate when reacted with methylamine (Table II). (Similar results have previously been reported for native enzyme but the product obtained from acid-denatured intermediate was not clearly indicated (3)). The 4H2O could be released by denaturation from a basic group on the protein or from FMNH2. The results with deazaFMN enzyme indicate that the α hydrogen of glutamate is transferred to deazaFMNH2 during intermediate formation and that this hydrogen is utilized

FIG. 8. Paper chromatography of tritium-labeled deazaFMN. Intermediate prepared from [α-3H]glutamate was denatured with 0.25 M formic acid and then chromatographed on a Dowex AG-1 column, as previously described (10). The column was washed with 0.5 M formic acid (α-hydroxyglutarate and amino acids elute in this fraction) and deazaFMN was then eluted with 10.0 M formic acid. Formic acid was removed by extraction with ether, the concentrated sample was applied in a 1-inch strip to a paper chromatogram and the chromatogram was developed in 5% NaOH 0.1 M in water. deazaFMN was located by fluorescence (A). The chromatogram was then cut into 1-cm strips, deazaFMN was eluted by shaking in 1.0 ml of water for 24 hours, and the radioactivity of each fraction determined by liquid scintillation counting (B).
TABLE II

| Intermediate | Treatment          | Products          | epm | Amino acid |
|-------------|--------------------|------------------|-----|-----------|
| Native enzyme | Acid denaturation  | 21,800           | 1,480  |
|             | Methylamine        | 6,590            | 17,000 |
| FMN-reconstituted enzyme | Acid denaturation  | 25,000           | 1,220  |
|             | Methylamine        | 7,310            | 18,000 |

Fig. 9. Paper chromatography of α-hydroxyglutarate using isooctanol ether-formic acid-water (90:70:3) as solvent. Tritium labeled α-hydroxyglutarate was isolated from deazaFMN enzyme intermediate precipitated with [α-3H]glutamate, carrier α-hydroxyglutarate was visualized by spraying with bromphenol blue (A), and radioactivity was located by elution (B), as described under “Methods.” The following results suggest that Spot I is α-hydroxyglutarate and Spot II is the lactone of α-hydroxyglutarate. Untreated samples of α-hydroxyglutarate yield Spot I; Spots I and II are obtained if the sample is reacted under conditions known to yield the lactone (22) or if the sample is subjected to the procedure used for the isolation of radioactive α-hydroxyglutarate from enzyme intermediate.

for the reformation of the α C—H bond during reaction of this intermediate with methylamine.

Kinetic Competence of the DeazaFMN Enzyme Intermediate—The pseudo-first order rate constants for formation and reaction of the deazaFMN enzyme intermediate are compared in Table III with the turnover number of the enzyme in the corresponding over-all reaction measured under the same conditions except that the substrate, absent in the half-reaction, is saturating in the over-all reaction. The rate of intermediate formation with glutamate is equal to the rate of the corresponding over-all reaction and the rate of reaction of the isolated intermediate with methylamine is considerably greater than the rate of the corresponding over-all reaction. These results indicate that the deazaFMN enzyme intermediate exhibits kinetic properties expected for a true catalytic reaction intermediate. The rapid rate of intermediate reaction, as measured by the rate of amino acid product formation, is consistent with the previously described rapid rate of reoxidation of reduced deazaFMN observed upon addition of methylamine or ammonia to substrate-reduced deazaFMN enzyme.

Formation of α-Hydroxyglutarate—An analysis of the reaction products obtained when deazaFMN enzyme intermediate prepared with [U-14C]glutamate is denatured with acid showed the presence of α-[14C]hydroxyglutarate in addition to α-[14C]ketoglutarate. When [α-3H]glutamate is used as substrate, the α-hydroxyglutarate formed contains tritium (Fig. 9). α Hydroxyglutarate is not an artifact arising during isolation of the enzyme intermediate since its formation can also be demonstrated fol-
The presence and absence of methylamine. Curves 1 and 2 show the disappearance of α-ketoglutarate and the formation of N-methylglutamate, respectively, observed during incubation of methylamine with deazaFMN enzyme intermediate prepared with (U-14C)glutamate. Curves 3 and 4 show the decay of α-ketoglutarate and the formation of amino acid (identified as glutamate), respectively, during incubation of the intermediate in the absence of methylamine. Reaction conditions and product identification were performed as described in the legend to Table I.

Following reaction of enzyme with [α-3H]glutamate without prior chromatography on a Sephadex G-25 column, as described under “Methods.” Since we have established that tritium is transferred from [α-3H]glutamate to deazaFMNH2, the formation of α-[3H]hydroxyglutarate could occur via a slow oxidation of [3H]-deazaFMNH2 by either enzyme-bound (unlabeled) α-ketoglutarate or an intermediate at the same oxidation level as α-ketoglutarate (3).

The formation of α-hydroxyglutarate by this mechanism is suggested by several observations. (a) A slow disappearance of α-ketoglutarate is observed when intermediate prepared with [U,14C]glutamate is incubated in the absence of methylamine. Unlike the rapid disappearance of α-ketoglutarate observed when the intermediate is incubated in the presence of methylamine, this slow decay of α-ketoglutarate cannot be accounted for by amino acid formation (Fig. 10), and free α-ketoglutarate is stable under these conditions. (b) The amount of α-ketoglutarate formed during reaction of deazaFMN enzyme with N-methylglutamate is stoichiometric with the amount of reduced deazaFMN produced, whereas the amount of methylamine, initially stoichiometric with reduced flavin, continues to increase after flavin reduction is apparently complete (Fig. 11). These results are consistent with the slow formation of α-hydroxyglutarate via Reactions 3 and 4.

\[
E \text{-deazaFMN} + N\text{-methylglutamate} \rightarrow E - \text{deazaFMNH}_2 + \text{glutaryl} + \text{methylamine} \tag{3}
\]

\[
E - \text{deazaFMNH}_2 + \text{glutaryl} \rightarrow E - \text{deazaFMN} + \alpha\text{-hydroxyglutarate} \tag{4}
\]

deazaFMN enzymes. The slow formation of methylamine in the absence of net flavin reduction is due to recycling of oxidized enzyme formed in Reaction 4. The decay of the enzyme intermediate via Reaction 4 is also consistent with the slow disappearance of α-ketoglutarate observed during incubation of the isolated intermediate in the absence of methylamine.

α-Hydroxyglutarate formation is not observed with FMN enzyme. The reason for this difference between FMN and deazaFMN enzymes remains unclear but may be due to a preferential decay of FMN enzyme intermediate via oxidation of reduced flavin by oxygen instead of the glutaryl residue.

**DISCUSSION**

A number of criteria have been presented in this paper which strongly suggest that N-methylglutamate synthetase, containing deazaFMN in place of FMN, catalyzes the conversion of glutamate and methylamine into N-methylglutamate and ammonia via the same two-step mechanism previously elucidated for native enzyme (3). These include (a) enzyme-bound deazaFMN is reduced by L-glutamate or N-methyl-L-glutamate but not by α-glutamate; (b) substrate-reduced deazaFMN enzyme is reoxidized by ammonia or methylamine; (c) a catalytically active deazaFMN enzyme intermediate can be isolated by Sephadex G-25 chromatography; (d) Incorporation of radioactivity into the isolated intermediate is observed following reaction with [U,14C]glutamate, [α-3H]glutamate, or N-[glutaryl-14C]methylglutamate but not N-[methyl-14C]methylglutamate; (e) the amount of radioactivity incorporated is approximately stoichiometric with the amount of deazaFMN reduced during intermediate formulation; (f) intermediate prepared with [U,14C]glutamate releases α-[U,14C]ketoglutarate when denatured with acid and N-[glutaryl-14C]methylglutamate when reacted with methylamine; (g) the rate of intermediate formation is equal to the rate of the over-all reaction, whereas the rate of the inter-
mediate reaction with amine substrate is greater than the rate of the over-all reaction.

Results obtained with intermediate prepared using [α-H]-glutamate (10) show that the α-hydrogen of glutamate is transferred to deazaFMNH₂ and that the same hydrogen is utilized for the reformation of the α C—H bond during reaction of the intermediate with amine substrate. The tritium in deazaFMNH₂ (released by acid denaturation of the intermediate or by phenol extraction) is nonexchangeable with solvent and less than 50% is lost during air oxidation, indicating that the α substrate hydrogen is probably attached to C-5 in deazaFMNH₂. A similar hydrogen transfer appears likely with native enzyme since other rates are different.

Shinkai and Bruice (8) during the reduction of the carbonyl group (25). Direct hydrogen transfer has also been observed by Benecke et al. (24) for microsomal n-amino acid oxidase, L-amino acid oxidase, and lactate oxidase.

Although our data provide the first direct demonstration of enzymic hydrogen transfer from substrate to flavin in enzymic reactions has been presented by Louie and Kaplan (23) for pyridine nucleotide transhydrogenase from Pseudomonas aeruginosa and by Drysdale et al. (24) for microsomal NADH-cytochrome b₅ reductase. Briistlein and Bruice (7) have shown hydrogen transfer from NADH to deazariboflavin catalyzed by NADH : FMN oxidoreductase from Beneckeia harvey (25). Direct hydrogen transfer has also been observed by Shinkai and Bruice (8) during the reduction of the carbonyl group of pyrroloxy by 1,5-dihydro-5-deaza-3,10-dimethylisoalloxazine.

Although our data provide the first direct demonstration of enzymic hydrogen transfer from substrate to flavin, the mechanism of this hydrogen transfer is not presently understood. Theoretical calculations indicate that the 5 position of flavins and deazaflavins has been reported (8, 32) and recent studies have provided evidence for carbanion formation in the reactions catalyzed by the enzymes (deaza)FMN and recent studies have provided evidence for the over-all reaction.

The reduction of certain carbonyl compounds by reduced flavins and deazaflavins has been reported (8, 32) and our observations suggest that the formation of α-hydroxyglutarate observed with deazaFMN enzyme also occurs via a slow reduction of enzyme-bound α-ketoglutarate (or an intermediate at the same oxidation level as α-ketoglutarate) by reduced deazaFMN.

REFERENCES

1. SHAW, W. V., TSAI, L., AND STADTMAN, E. R. (1968) J. Biol. Chem. 243, 935-945

2. POLLOCK, R. J., AND HERSH, L. B. (1971) J. Biol. Chem. 246, 4737-4743

3. POLLOCK, R. J., AND HERSH, L. B. (1973) J. Biol. Chem. 248, 6724-6733

4. O'BRIEN, D. E., WINSTECK, L. T., AND CHENG, C. C. (1970) J. Heterocyclic Chem. 7, 90-105

5. EDMONDSON, D. E., BARMAN, B., AND TOLLIN, G. (1972) Biochemistry 11, 1133-1144

6. MOLLER, F., AND MASSEY, V. (1969) J. Biol. Chem. 244, 4001-4016

7. BRITZELM, M., AND BRUCE, T. C. (1972) J. Am. Chem. Soc. 94, 6548-6549

8. SHINKAI, S., AND BRUICE, T. C. (1973) J. Am. Chem. Soc. 95, 7525-7528

9. SUN, M., AND SONG, P. S. (1973) Biochemistry 12, 4663-4669

10. JOHNS, M. S., AND HERSH, L. B. (1974) J. Am. Chem. Soc. 96, 4032-4041

11. FLEXSER, L. A., AND FARKAS, W. G. (1952) U. S. Patent 2,610,170; Chem. Abstr. 48, 8781g

12. MASSEY, V., AND SWOBODA, B. E. P. (1963) Biochem. Z. 330, 474-484

13. LAYNE, E. (1957) Methods Enzymol. 3, 447-454

14. STRITTMATTER, P. (1961) J. Biol. Chem. 236, 2329-2339

15. BEINERT, H. (1960) in The Enzymes (BOYER, P. D., ed) Vol. II, pp. 413-429, Academic Press, New York

16. FROST, A. A., AND PEARSON, R. G. (1961) Kinetics and Mechanism, 2nd Ed, pp. 49-50, John Wiley and Sons, Inc., New York

17. LARGO, P. J., EADE, R. R., AND MURDEN, D. J. (1969) Anal. Biochem. 39, 402-407

18. BERGMeyer, H. U., AND BENRT, E. (1965) in Methods of Enzymatic Analysis (BERGMeyer, H. U., ed) pp. 324-327, Academic Press, New York

19. BLOCK, R. J., DURRUM, E. L., AND ZWEIG, G. (1968) A Manual of Paper Chromatography and Paper Electrophoresis, 2nd Ed, pp. 235-239, Academic Press, New York

20. WILLIAMSON, J. R., AND CORREY, B. E. (1969) Methods Enzymol. 13, 455-458

21. RANDERATH, K. (1970) Anal. Biochem. 34, 188-205

22. BLEWIES, A. S., REEVES, H. C., AND AIL, S. J. (1967) J. Bacteriol. 94, 1560-1564

23. LOUIE, D. D., AND KAPLAN, N. (1970) J. Biol. Chem. 245, 5691-5699

24. DRYSDALE, G. R., SPIEGEL, M. J., AND STRITTMATTER, P. (1961) J. Biol. Chem. 236, 2329-2339

25. FISHER, J., AND WALSH, C. (1974) J. Am. Chem. Soc. 96, 4345-4346

26. WALSH, C. T., SCHONBRUNN, A., AND ABELES, R. H. (1971) J. Biol. Chem. 246, 6855-6865

27. WALSH, C. T., KRODEL, E., MASSEY, V., AND ABELES, R. H. (1973) J. Biol. Chem. 248, 1946-1955

28. WALSH, C., LOCKRIDGE, O., MASSEY, V., AND ABELES, R. (1973) J. Biol. Chem. 248, 7049-7054

29. PORTER, D. J. T., VOET, J. G., AND BRIGHT, H. J. (1973) J. Biol. Chem. 248, 4410-4416

30. PAGE, D. S., AND VANetten, R. L. (1971) Biochim. Biophys. Acta 257, 19-31

31. PAGE, D. S., AND VANetten, R. L. (1971) Biochem. 1, 361-373

32. BLANKENHORN, G., GHISLA, H., AND HEMMERICH, P. (1972) Z. Naturforsch. 27b, 1038-1040
N-Methylglutamate synthetase. Substrate-flavin hydrogen transfer reactions probed with deazaflavin mononucleotide.

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