Identification of Acetyl Phosphate as the Product of Clostridial Glycine Reductase: Evidence for an Acyl Enzyme Intermediate†

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Received October 26, 1988; Revised Manuscript Received January 25, 1989

ABSTRACT: It has been reported [Tanaka, H., & Stadtman, T. C. (1979) *J. Biol. Chem. 254*, 447–452] that glycine reductase from *Clostridium sticklandii* catalyzes the reaction glycine + ADP + P_i + $2e^-$ → acetate + ATP + NH_4^+. Glycine reductase consists of three proteins, designated A, B, and C. Only A has been purified to homogeneity. A dithiol serves as an electron donor. We find that ADP is not essential for the reaction and that in its absence acetyl phosphate is formed. Upon further purification of components B and C, an acetate kinase activity can be separated from both proteins. This observation establishes that acetate kinase activity is not an intrinsic property of glycine reductase, and therefore the reaction catalyzed by glycine reductase is glycine + P_i + $2e^-$ → acetyl phosphate + NH_4^+. Experiments with [^{14}C]glycine and unlabeled acetate show that free acetate is not a precursor of acetyl phosphate. When glycine labeled with ^{18}O is converted to product, ^{18}O is lost. The ^{18}O content of unreacted glycine remains unchanged after approximately 50% is converted to product. We propose that an acyl enzyme, most probably an acetyl enzyme, is an intermediate in the reaction and that the acetyl enzyme reacts with P_i to form acetyl phosphate. A mechanism is proposed for the formation of the acetyl enzyme.

The clostridial glycine reductase complex is composed of three components: protein A and two membrane-associated proteins, B and C (Tanaka & Stadtman, 1979). Protein A contains a selenocysteine residue (Cone et al., 1976). Protein B is sensitive to carbonyl reagents, suggesting that a carbonyl cofactor[1] is necessary for catalytic activity (Turner & Stadtman, 1973). Protein A has been purified to near homogeneity. B and C are still relatively impure. It has been reported that the enzyme complex catalyzes the reaction

$$^+NH_3CH_2COO^- + 2e^- + P_i + ADP \rightarrow CH_3COO^- + NH_4^+ + ATP \quad (1)$$

In vitro, DTT can serve as an electron donor for the system. One equivalent of ATP is synthesized from 1 equiv of orthophosphate and 1 equiv of ADP concomitant with the reduction of glycine (Tanaka & Stadtman, 1979). However, in the absence of ADP the amount of acetate formed is one-third that formed in the presence of ADP (Stadtman et al., 1958; Barnard & Akhtar, 1979).

The reaction catalyzed by glycine reductase is a fascinating reaction for several reasons. The mechanism of reduction of glycine to acetate is not at all obvious. It would be extremely difficult to carry out this reaction without the use of enzymes. Additionally, this reaction gives rise to a high-energy compound. We have, therefore, undertaken an investigation of this reaction. In the course of this work, it became apparent that the product of the reaction is acetyl phosphate, and not acetate and ATP as originally reported. We also have investigated the fate of the carboxyl oxygens of glycine during glycine reduction in order to characterize the mechanism of action of glycine reductase.

Materials and Methods

Reagents

ADP, AMP, Tricine,[2] $(NH_4)_2SO_4$, benzoic anhydride, CoASH, acetyl phosphate, alkaline phosphatase, and phosphotransacetylase were purchased from Sigma. Acetyl chloride and 18-crown-6 were purchased from Aldrich. *p*-Phenylphenacyl bromide from K and K Laboratories was recrystallized twice from ethyl acetate and petroleum ether prior to use. DTT was obtained from Research Organics. KBH_4 was purchased from Metal Hydrides. [^{18}O]Water (98 atom %) was purchased from ICN or Cambridge Isotope Laboratories. [1-^{14}C]Glycine (40 μCi/μmol) was from New England Nuclear, and [1,2-^{14}C]glycine (100 μCi/μmol) was from ICN. [^{14}C]Glycine was purified by chromatography on Dowex 50-X8 (H^+) resin and eluted with 3 N HCl prior to use. [2,8-^{3}H]ADP trisodium salt (25 Ci/mmol), from New England Nuclear, was >96% radiochemically pure (anion-exchange HPLC) and was used without further purification.

Assays and Analytical Procedures

Mass Spectrometry. Mass spectra were obtained with a Hewlett-Packard 5985B mass spectrometer using a direct inlet system. Samples were ionized with chemical ionization using methane gas ($\sim 5 \times 10^{-4}$ mmHg) and an ion source temperature of 150 °C. The ^{18}O content of the derivatives was determined from the signal intensities of the molecular ion (M), M + 2, and M + 4 and was uncorrected for natural ^{18}O abundance (0.2%). The fragmentation patterns of the samples agreed with those of the pure compounds.

Glycine Reductase. The activity of reconstituted glycine reductase (proteins A, B, and C) was assayed by measuring the amount of ^{14}C anionic product formed from [^{14}C]glycine (Tanaka & Stadtman, 1979). The standard assay mixture contained 60 mM Tricin/KOH, pH 8.1, 20 mM K_2HPO_4, 4 mM ADP, 4 mM AMP, 8 mM $MgCl_2$, 40 mM DTT, and 40 mM [^{14}C]glycine (5000–6000 cpm/μmol), in a final volume of 250 μL. AMP was included in the assay mixture because

†This work was supported by National Science Foundation Grant DMB 8645903.

[1] Preliminary experiments with [^{3}H]$NaBH_4$ establish the presence of covalently bound pyruvate in protein B (Robert A. Arkowitz and Robert H. Abeles, unpublished data).

[2] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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of a highly active adenylate kinase contaminant that was present in all but highly purified enzymes (Tanaka & Stadtman, 1979). The reaction was started by addition of DTT. The assay was carried out in a tube stoppered with a serum cap under an atmosphere of argon for 90 min at 30 °C. Amounts of proteins (A, B, and C) are indicated with each experiment. The reaction was terminated with the addition of an equal volume of ethanol. After removal of the precipitate, 350 μL of the supernatant fluid was loaded onto a Dowex 50-X8 (H^+) column (0.6 × 2 cm), which was then washed with 2 × 1 mL of H_2O. Radioactivity in the effluent was determined by using ACS counting fluid (Amersham).

Acetate Kinase. Acetate kinase activity, in some experiments, was assayed by the formation of [^{3}H]ATP from acetyl phosphate and [^{3}H]ADP. [^{3}H]ATP formation was determined by using the standard assay mixture with 40 mM acetyl phosphate in place of glycine and approximately 1.3 × 10^6 dpm/μmol [^{3}H]ADP. After 90 min of incubation at 30 °C, 0.1 μmol of ATP was added to 220 μL of the reaction solution, followed by 80 μL of 70% perchloric acid. The supernatant fluid was neutralized with concentrated KOH and injected onto a 4.6 × 250 mm Alltech anion-exchange Spherisorb SAX HPLC column. The column was eluted with 50 mM KH_2PO_4 for 5 min, followed by a 0–430 mM KH_2PO_4 gradient over a 10-min period. Thereafter, an isocratic elution was continued at 430 mM KH_2PO_4. The flow rate was 0.8 mL/min. AMP eluted after 10 min, ADP after 16 min, and ATP after 18 min. Fractions (0.5 min) were collected and used for determination of radioactivity.

Enzyme Isolation

Protein A. Protein A and the glycine reductase fraction (B and C activity) were isolated from frozen *C. sticklandii* cells (160 g) by a modification of the procedure described (Turner & Stadtman, 1973).

In place of the final DEAE-cellulose chromatography, protein A was chromatographed twice on a 10 × 100 mm FPLC Pharmacia Mono Q cation-exchange column. In a typical purification, 2 mg of KBH_4 was added to 54 mg of protein in 4.5 mL of 100 mM K_2HPO_4, 1 mM DTT, and 1 mM MgK_2EDTA, pH 7.2, which was incubated at 4 °C for 2 h. This sample was then loaded onto the column. The column was equilibrated and eluted with the previously mentioned buffer for 7.5 min. A 0–350 mM NaCl gradient was developed over 5 min, followed by isocratic flow at 350 mM NaCl for 2.5 min, and finally a 20-min gradient to 450 mM NaCl was developed. The flow rate was 4 mL/min. Protein A eluted at approximately 15 min (350 mM NaCl). Protein A was typically >99% homogeneous as judged by SDS gel electrophoresis. The specific activity of protein A in the presence of saturating amounts of the glycine reductase fraction was 50–100 μmol of anionic product 90 min^{-1} (mg of protein)$^{-1}$. This variability in apparent specific activity of protein A is most likely due to variability in the preparations of the glycine reductase fraction. These specific activities are in agreement with values observed previously: 23–93 μmol 90 min^{-1} (mg of protein)$^{-1}$ (Turner & Stadtman, 1973; Stadtman, 1966). Protein A was stored frozen at −70 °C.

Glycine Reductase Fraction. After separation from protein A on a Whatman DE-23 column (10 × 7 cm) and ammonium sulfate precipitation (30–50% saturation), following the procedure of Tanaka & Stadtman (1979), the glycine reductase fraction was further purified by gel filtration chromatography. The ammonium sulfate pellet (1.2 g of protein) was resuspended in 20 mM K_2HPO_4, 1 mM DTT, and 1 mM MgK_2-EDTA, pH 7.2 (buffer A), total volume 28 mL. This was then loaded onto a 2.6 × 88 cm Bio-Gel A-0.5m (Bio-Rad) column equilibrated with buffer A. The column was eluted with buffer A at a flow rate of 84 mL/h. The fractions containing both B and C activity (assayed by reconstitution with excess protein A) were pooled and concentrated with a Diaflow PM-10 (Amicon) membrane. This concentrate was loaded onto a 2.5 × 65 cm Sephacryl S-300 (Pharmacia) column equilibrated and eluted with buffer A (flow rate 60 mL/h). The fractions containing both B and C activity were pooled and concentrated with a Diaflow PM-10 membrane. This material will be referred to as the glycine reductase fraction and contains proteins B and C. The specific activity of the glycine reductase fraction, in the presence of saturating amounts of protein A, was 3 μmol of anionic product 90 min^{-1} (mg of protein)$^{-1}$. The concentrated glycine reductase fraction was stored anaerobically at 4 °C.

Resolution of Proteins B and C. The glycine reductase fraction was prepared as described in the previous section by using DE-23 chromatography and ammonium sulfate precipitation. Following resuspension of the ammonium sulfate pellet (3.0 g) in 5 mM K_2HPO_4, 1 mM DTT, and 1 mM MgK_2EDTA, pH 7.2 (buffer B), containing 1.2 M $(NH_4)_2SO_4$, pH 7.2, the protein solution was dialyzed against this buffer. The protein solution (52 mL) was then chromatographed on a 5 × 88 cm Sephacryl S-300 column equilibrated in buffer B containing 1.2 M $(NH_4)_2SO_4$. The column was eluted with the same buffer (flow rate 150 mL/h). The glycine reductase fraction (proteins B and C) eluted predominantly in the void volume. The pooled glycine reductase fraction (106 mg of protein in 177 mL) was loaded onto a 2.5 × 42 cm Phenyl-Sepharose CL-4B (Pharmacia) column equilibrated in buffer B containing 1.2 M $(NH_4)_2SO_4$. This column was used to separate components B and C. The column was washed with 200 mL of buffer B containing 1.2 M $(NH_4)_2SO_4$ and 200 mL of buffer B containing 0.5 M $(NH_4)_2SO_4$, and a 1-L gradient was developed from 0.5 M $(NH_4)_2SO_4$ in buffer B to buffer B containing no $(NH_4)_2SO_4$ (flow rate 120 mL/h). The column was subsequently washed with buffer B until the OD_{280} decreased to background levels and was finally eluted with water. Protein B typically eluted between 0.35 and 0.15 M $(NH_4)_2SO_4$, and protein C eluted in the H_2O wash to which K_2HPO_4 and DTT, pH 7.2, were added to final concentrations of 5 and 1 mM, respectively. Components B and C were concentrated with a Diaflow PM-10 membrane and, unless otherwise indicated, used without further purification. Protein B and C activities were each assayed by reconstitution in the presence of saturating amounts of the other two proteins. The specific activity for resolved protein B (with saturating amounts of protein A and protein C) was typically 17–33 μmol of anionic product 90 min^{-1} (mg of protein)$^{-1}$. Resolved component C had a specific activity of approximately 40 μmol 90 min^{-1} (mg of protein)$^{-1}$. Although both preparations were not homogeneous, as judged by polyacrylamide gel electrophoresis, these specific activities for components B and C are comparable to those reported previously, 5.5 μmol 90 min^{-1} (mg of protein)$^{-1}$ for B and 1.9 μmol 90 min^{-1} (mg of protein)$^{-1}$ for C, using detergents to separate B and C (Tanaka & Stadtman, 1979). Component B was stored frozen at −20 °C and component C at 4 °C in buffer B with 1.2 M $(NH_4)_2SO_4$.

Further Purification of Component B. Concentrated protein B was further purified by FPLC Mono Q cation-exchange chromatography. The flow rate was 2 mL/min. Protein B (17.2 mg in 2.1 mL of buffer B) was loaded onto the column (5 × 50 mm) equilibrated and eluted with buffer B for 5 min. A 0–104 mM K_2HPO_4 gradient, pH 7.2, was developed over

10 min, followed by a 20-min gradient to 225 mM K_2HPO_4. Protein B eluted at approximately 134 mM K_2HPO_4, as assayed by reconstitution with component C and protein A. The pooled protein B activity was concentrated by using an Amicon centriprep.

Characterization of Reaction Products

Reaction of Acetyl Phosphate, Derived from Glycine, with Phosphotransacetylase and CoASH. Under standard assay conditions (−ADP/AMP), 44 μg of protein A, 375 μg of component B, and 86 μg of component C were incubated for 2 $^1/_2$ h at 30 °C. Acetyl phosphate (3.2 μmol) in an equal volume was added, and the sample was deproteinized with an Amicon centricon. The filter was washed with an additional 0.5 mL of H_2O. From a 250-μL aliquot of the combined filtrate acetyl phosphate was isolated by using a Dowex 1-X8 (formate) column (Figure 1). To a 0.5-mL aliquot of the filtrate containing acetyl phosphate were added 2 μmol of CoASH (80 μL) and 100 units of *Clostridium kluyveri* phosphotransacetylase. The reaction was allowed to proceed for 1 h at 25 °C. The protein was then precipitated with 50 μL of 70% perchloric acid. The supernatant fluid was loaded onto a Waters C-18 Sep-Pak cartridge. The cartridge was washed with 10 × 1 mL of H_2O, and acetyl-CoA was eluted with 50% MeOH. The MeOH was removed by rotary evaporation under reduced pressure, and C-18 HPLC analysis was performed on a 3.9 × 300 mm Waters μBondapak C-18 column. The column was eluted isocratically with 10% MeOH in 20 mM ammonium acetate, pH 5.8, with a flow rate of 1 mL/min. One-minute fractions were collected and counted. Acetyl-CoA eluted at 9 min.

Reaction of [^{18}O]Glycine and [^{18}O]Acetate with Glycine Reductase. Under standard assay conditions (−ADP/AMP), 88 μg of protein A, 0.3–1.1 mg of component B, and 189 μg of component C were incubated in the presence of 40 mM [^{18}O]glycine [with (5–10) × 10^4 cpm of [^{14}C]glycine added] or 40 mM [^{18}O]acetate and 25 units of *Escherichia coli* alkaline phosphatase. The reaction was incubated at 30 °C until 31–50% of the glycine was converted to acetate (90–120 min). The reaction was then quenched with 80 μL of 1 N HCl. The protein precipitate was removed by centrifugation, and the supernatant fluid was bulb-to-bulb distilled. The pH of the distillate, which contained acetic acid, was adjusted to 10–11 with 20 μL of 1 N KOH. The residue was saved for the isolation of unreacted glycine. The distillate was then lyophilized and subsequently derivatized with *p*-phenylphenacyl bromide as described subsequently. The glycine in the redissolved residue was purified by high-voltage paper electrophoresis. The residue was electrophoresed on 30 × 57 cm Whatmann 3MM paper in pH 1.9 H_2O–formic acid–acetic acid (45:1:4) buffer at 61 V/cm for 30 min. Glycine standards were visualized by ninhydrin and migrated approximately 20 cm toward the positive electrode. The glycine was eluted with water (90% recovery as determined by radioactivity). The eluted glycine was lyophilized and converted to *N*-benzoylglycine as described subsequently.

Synthetic Procedures

Synthesis of [^{18}O]Glycine and [^{18}O]Acetate. Glycine hydrochloride (75 mg, 0.7 mmol) was dissolved in 1 mL of [^{18}O]H_2O (98 atom %) and incubated for 2 days in vacuo at approximately 80 °C. Excess [^{18}O]water was removed by distillation, and the sample was dried over P_2O_5 under vacuum. The labeled glycine migrated as a single ninhydrin-positive spot on Eastman cellulose TLC (13254) developed in *t*-BuOH–acetic acid–H_2O (9:1:4). Mass spectral analysis of the *N*-benzoyl derivative showed that the carboxyl group contained 5.1% $^{16}O_2$, 31.9% $^{18}O^{16}O$, and 63.0% $^{18}O_2$.

Acetyl chloride (71 μL, 1 mmol) was added to 0.5 mL of [^{18}O]water (98 atom %) and incubated for 5 days in vacuo at approximately 80 °C. The reaction was made alkaline with the addition of 2 mmol of KOH and lyophilized to remove water. The concentration and purity (>95%) of acetate in the sample were determined by Aminex organic acid HPLC analysis using a Waters refractive index detector. Mass spectral analysis of the *p*-phenylphenacyl derivative showed that the carboxyl group contained 9.5% $^{16}O_2$, 38.9% $^{18}O^{16}O$, and 51.6% $^{18}O_2$.

p-Phenylphenacyl Acetate. Acetate was derivatized for mass spectral analysis following the procedure of Cane et al. (1982). *p*-Phenylphenacyl acetate was purified by silica gel preparative layer chromatography (Analtech GF plates with fluorescent indicator) developed in benzene to give 3.2 μmol of *p*-phenylphenacyl acetate. Yields ranged between 63% and 74% and were determined by radioactivity. The purified *p*-phenylphenacyl acetate migrated as a single UV fluorescent spot on silica gel TLC (Merck) developed in benzene, R_f 0.2; ^{1}H NMR (Varian XL-300, $CDCl_3$) δ 2.16 (s, CH_3, 3 H), 5.28 (s, CH_2, 2 H), 7.3–8.0 (m, Ar CH, 9 H).

N-Benzoylglycine. Glycine was benzoylated with benzoic anhydride. The progress of the reaction was followed by silica gel TLC (visualized by UV light) developed in benzene–acetic acid–ethyl acetate (6:1:13); R_f (*N*-benzoylglycine) 0.2. The reaction was stopped by the addition of 1 N HCl to a final pH of 1–2. The product was extracted with 8 × 1 mL portions of ethyl acetate. The combined extracts were brought to dryness under reduced pressure. The residue was taken up in 200 μL of benzene–acetic acid–ethyl acetate (6:1:13) and chromatographed in the same solvent system on a 0.6 × 6.5 cm flash silica gel (Merck) column (1 g). Fractions (approximately 0.5 mL) were collected and analyzed by TLC. The fractions containing *N*-benzoylglycine were pooled, and the solvent was removed under reduced pressure followed by lyophilization. We obtained 2.7 μmol of *N*-benzoylglycine (50% yield, as determined by radioactivity). The purified product migrated as a single UV fluorescent spot on silica TLC developed in the benzene–acetic acid–ethyl acetate solvent system; ^{1}H NMR (Varian XL-300, acetone-d_6) δ 4.04 (d, J = 6 Hz, CH_2, 2 H), 7.3–7.9 (m, Ar CH, 5 H), 7.86 (br s, NH, 1 H).

Results and Discussion

It was reported that glycine reductase, reconstituted from proteins A, B, and C, catalyzes the reaction shown in eq 1. Initially we carried out experiments to determine the nature of products formed in the absence of ADP. The reaction was allowed to proceed in the presence and absence of ADP, under standard assay conditions, with 39 μg of protein A and 2 mg of the glycine reductase fraction. When product formation was assayed on a Dowex 50-X8 (H^+) column, 2.5 μmol of anionic material was detected. The reaction mixture was also analyzed on an Aminex HPLC column (7.8 × 300 mm). The column was eluted isocratically with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. A single radioactive peak was found that cochromatographed with acetate. When ADP was omitted from the reaction mixture, 0.78 μmol of anionic product was found, consistent with previous observations (Stadtman et al., 1958; Barnard & Akhtar, 1979). Upon chromatography on an Aminex HPLC column, two radioactive peaks were detected. One contained 0.53 μmol of radioactive material (based on the specific activity of glycine) that migrated near the solvent front and the other contained 0.13 μmol of radioactive

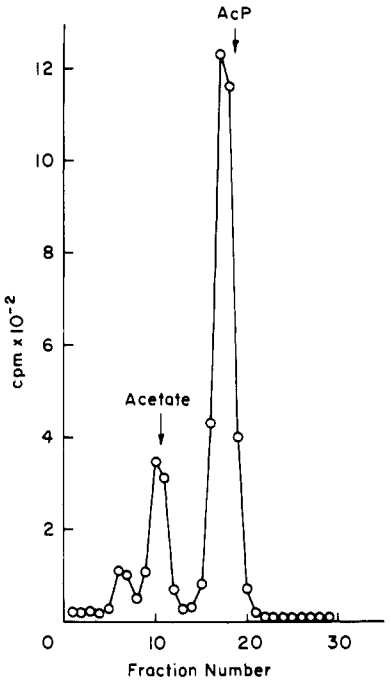


FIGURE 1: Analysis of reaction products. The reaction was carried out under standard assay conditions (−ADP/AMP) with 44 μg of selenoprotein A, 375 μg of component B, and 86 μg of component C. A 250-μL aliquot of deproteinized assay solution was loaded onto a 0.6 × 3.5 cm Dowex 1 (formate) column, which was washed with 10 × 0.8 mL of H_2O to remove [^{14}C]glycine and eluted with a 30-mL 0–0.5 M ammonium formate gradient; 1.0-mL fractions were collected. Acetate eluted at approximately 0.18 M ammonium formate (fractions 10 and 11), and acetyl phosphate eluted at approximately 0.31 M ammonium formate (fractions 16–20).

material that comigrated with acetate. In order to characterize this product formed in the absence of ADP, a parallel experiment was carried out under identical conditions except 78 μg of protein A and 4 mg of the glycine reductase fraction were used. The compound that migrated near the solvent front on an Aminex HPLC column was again isolated. An aliquot (700 μL) containing the unknown radioactive compound (0.18 μmol based on glycine specific activity) was brought to pH ~8 with 1 N KOH. *E. coli* alkaline phosphatase (1.6 units) was added, and the sample was incubated at 37 °C for 1 h. The reaction was terminated with 140 μL of 10% perchloric acid, and 800 μL of the supernatant fluid was analyzed by Aminex HPLC. Only acetate (0.16 μmol) was detected. In a control reaction without alkaline phosphatase, two radioactive peaks were detected by Aminex HPLC, one containing 0.04 μmol of radioactive material (based on glycine specific activity) that migrated near the solvent front and the other containing 0.12 μmol of radioactive material that comigrated with acetate. These results indicate that this unknown radioactive material is both acid labile and sensitive to alkaline phosphatase treatment. These observations suggest that, in the absence of ADP, glycine reductase converts glycine to acetyl phosphate.

The following experiment was done to confirm the formation of acetyl phosphate: A reaction was carried out under the conditions described in Figure 1, and at the end of the reaction period an aliquot of the deproteinized reaction mixture was chromatographed on a Dowex 1 (formate) column. Two radioactive peaks were observed. One peak cochromatographed with acetyl phosphate and the other with acetate (Figure 1). To another aliquot of the reaction mixture containing the putative acetyl phosphate were added phosphotransacetylase and CoASH. Stoichiometric conversion to acetyl-CoA was detected upon chromatography on C-18 HPLC. These results

Table I: Requirements for Acetyl Phosphate Formation[a]

enzyme component(s)	acetyl phosphate[b] (μmol)
protein A	0.0
protein B	0.0
protein C	0.0
A, B, and C	0.6
A, B, C, DTT omitted	0.02
A, B (KBH_4 inactivated),[c] and C	0.0

[a] Standard assay conditions with additions as indicated using 66 μg of A, 750 μg of B, and 170 μg of C. Acetyl phosphate determined by Dowex 1 chromatography (Figure 1). [b] Detection limit for acetyl phosphate is 0.01 μmol. [c] B preincubated with ~1 mg of KBH_4 for 10 min.

confirm that glycine reductase produces acetyl phosphate when ADP is omitted.

Table I shows that all three glycine reductase components are necessary for acetyl phosphate synthesis. In addition, when the glycine reductase fraction was preincubated with KBH_4, which inactivates component B (Tanaka & Stadtman, 1979), no acetyl phosphate was produced, indicating that glycine reductase activity is necessary for acetyl phosphate synthesis.

A possible reaction sequence through which acetyl phosphate could be derived is

$$E + \text{glycine} + P_i + 2e^- \rightarrow E\sim P + \text{acetate} + NH_4^+ \quad (2)$$

$$E\sim P + ADP \rightarrow E + ATP$$

$$E\sim P + \text{acetate} \rightarrow E + \text{acetyl}\sim P$$

According to this reaction sequence, a phospho enzyme is formed, which can react with either ADP or acetate from solution. This is consistent with the observation that, in the presence of ADP, little or no acetyl phosphate is found. To test this reaction sequence, the reduction of glycine was carried out in the presence of 40 mM unlabeled acetate under standard assay conditions (−AMP/ADP) with 117 μg of protein A and 6 mg of the glycine reductase fraction. Acetyl phosphate was isolated by Dowex 1 (formate) chromatography as described in Figure 1 and determined according to Lipmann and Tuttle (1945). Unlabeled acetate did not decrease the specific activity (5.9×10^3 cpm/μmol) or affect the amount of acetyl phosphate formed (0.54 μmol). This observation shows that free acetate is not a precursor of acetyl phosphate.

Formation of ATP and Acetate. The experiments cited above establish that in the absence of ADP acetyl phosphate is formed. In the presence of ADP, ATP is produced. How is ATP formed? A possible mechanism is shown in eq 2. Another possibility is that ATP is produced by a kinase (eq 3), which copurifies with one or more of the glycine reductase components.

$$\text{glycine} + 2e^- + P_i \rightarrow \text{acetyl phosphate} + NH_4^+ \quad (3)$$

$$\text{acetyl phosphate} + ADP \rightarrow ATP + \text{acetate}$$

Proteins A, B, and C, separately, were assayed for acetate kinase activity. This activity was determined by measuring the formation of [^{3}H]ATP from acetyl phosphate and [^{3}H]-ADP. Proteins B and C showed acetate kinase activity. Both proteins phosphorylated 1 μmol of ADP only in the presence of acetyl phosphate (10 μmol) in a 90-min incubation. Protein A had no detectable activity under these conditions.

We found that acetate kinase activity could be separated from protein B (Figure 2). Similarly, it was possible to separate component C from acetate kinase activity (Figure 3).

The products obtained when glycine reductase is reconstituted by combining protein A with proteins B and C (purified as described in Figures 2 and 3) are shown in Table II. When

Table II: Acetyl Phosphate Formation with Highly Purified Glycine Reductase[a]

enzyme	ADP in assay (mM)	acetate (μmol)	AcP (μmol)	total (μmol)
A, B, C	8	0.48	0.0[b]	0.48
A, B, C (highly purified)	8	0.19	0.26	0.45
A, B, C (highly purified)	0	0.06	0.38	0.44

[a] Standard assay conditions with additions as indicated using either 56 μg of A, 52 μg of B, and 17 μg of C or 56 μg of A, 23 μg of B, and 13 μg of C of highly purified protein. Acetyl phosphate and acetate were analyzed by Dowex 1 (formate) chromatography as described in Figure 1. [b] Detection limit for acetyl phosphate is 0.01 μmol.

glycine reductase was reconstituted from its components, which were not purified by the procedure described in Figures 2 and 3, no acetyl phosphate formation was detected in the presence of ADP. When glycine reductase is reconstituted with proteins B and C, which had been purified by HPLC gel filtration, 60% of the total reaction product is acetyl phosphate in the presence of ADP. Residual acetate kinase activity in the purified individual components can account for the acetate formation in the presence of ADP. The small amount of acetate detected in absence of ADP is probably due to the hydrolysis of acetyl phosphate. We conclude that glycine reductase reconstituted with proteins A, B, and C carries out the reaction shown in eq 4. The previously observed formation of ATP is due to

$$\text{glycine} + P_i + 2e^- \rightarrow \text{acetyl}\sim P + NH_4^+ \qquad (4)$$

the presence of an acetate kinase, which copurified with proteins B and C.

Acyl Group Activation. The formation of acetyl phosphate from glycine requires that at some intermediate stage in the reaction "activated" glycine or phosphate participates. We have described experiments that are inconsistent with phospho enzyme formation. Experiments were also carried out to determine whether an intermediate exists containing glycine esterified with a nucleophile at the active site (activated glycine). The conversion of glycine to acetyl phosphate was carried out with glycine labeled in the carboxyl group with ^{18}O. The reaction was done in the presence of alkaline phosphatase to convert the product, acetyl phosphate, to acetate in order to prevent possible irrelevant reactions of acetyl phosphate. The reaction was allowed to proceed until approximately 50% of glycine was converted to acetate. A control experiment was carried out under identical conditions except glycine was replaced by [^{18}O]acetate. Acetate was then isolated and derivatized, and its ^{18}O content was determined. The results are summarized in Table III. The data show that ^{18}O from the carboxyl group is lost in the conversion of glycine to acetate (acetyl phosphate). No significant loss of ^{18}O occurs from acetate under identical conditions. The most reasonable explanation for this loss of ^{18}O is the intermediate formation of an acyl enzyme. These data do not rule out the possibility of acetyl phosphate formation from the attack of an inorganic phosphate oxygen atom on an acetate carboxyl group dis-

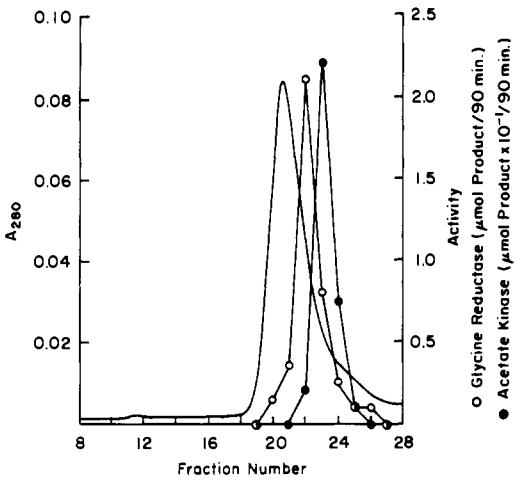


FIGURE 2: HPLC gel filtration chromatography of component B. A total of 100 μL of B (380 μg) from the Mono Q column was loaded onto a 7.5 × 300 mm Beckman TSK4000SW HPLC column eluted with 100 mM K_2HPO_4, 1 mM DTT, and 1 mM MgK_2EDTA, pH 7.2, at a flow rate of 0.5 mL/min. One-minute fractions were collected; 50 μL was assayed for acetate kinase activity (●) and 100 μL assayed for glycine reductase activity (○). Acetate kinase activity was determined in a reaction mixture consisting of 110 mM K_2HPO_4, 110 mM potassium acetate, 11 mM ATP, 22 mM $MgCl_2$, 1 mM DTT, pH 7.2, and 220 mM freshly neutralized NH_2OH–HCl in a 900-μL volume. After 90 min at 30 °C, 600 μL of 10% $FeCl_3$ in 0.7 N HCl was added to terminate the reaction, and absorbance at 540 nm was measured. Acetohydroxamic acid was used to generate standard curves. Activities reported are for 500-μL fractions.

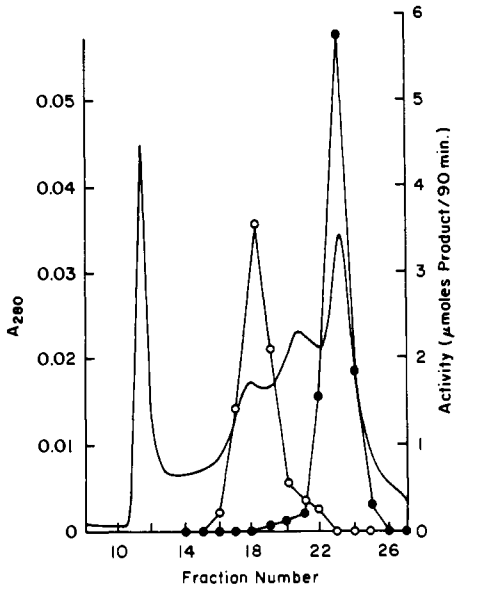


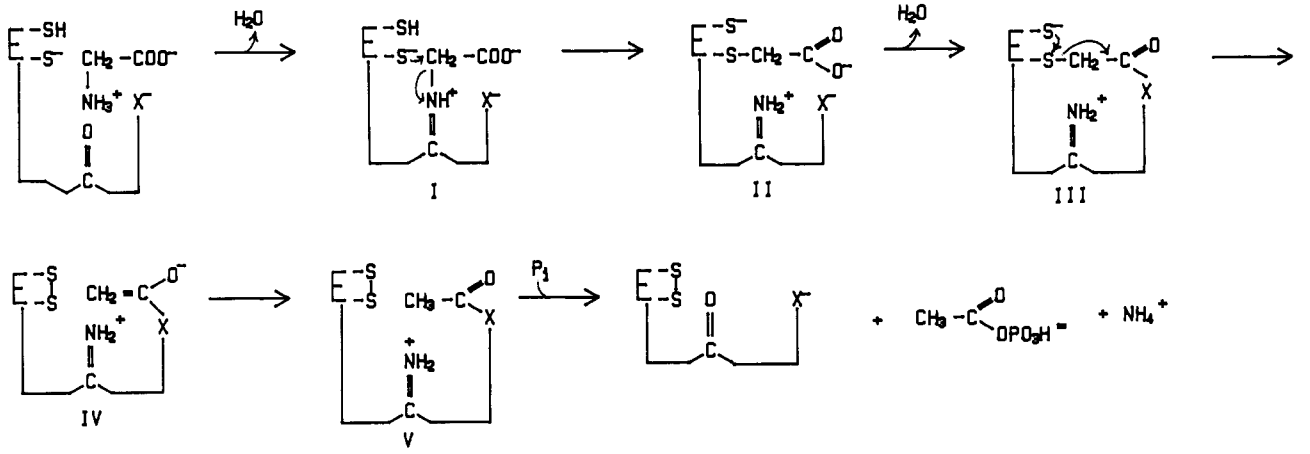
FIGURE 3: HPLC gel filtration chromatography of component C. A total of 50 μL (430 μg) of C from the Phenyl-Sepharose column was loaded onto a TSK4000SW column eluted as described in Figure 2. One-minute fractions were collected, and 100 μL was assayed for acetate kinase activity (●) (as described in Figure 2) and for glycine reductase activity (○). Activities reported are for 500-μL fractions.

Table III: ^{18}O Content of Unreacted Glycine and Acetate Derived from Glycine[a]

	% intensity					
	N-benzoylglycine			*p*-phenylphenacyl acetate		
reaction components	M, 180 (0)[b]	M + 2, 182 (1)	M + 4, 184 (2)	M, 275 (0)	M + 2, 277 (1)	M + 4, 279 (2)
A, B, C; 10 μmol of [^{18}O]glycine	7.3	33.1	59.6	74.0	24.6	*c*
A, B, C; 10 μmol of [^{18}O]acetate				15.7	38.1	46.2

[a] Standard assay conditions (−AMP/ADP) with 25 units of *E. coli* alkaline phosphatase added. [^{18}O]Glycine was 5.1% $^{16}O_2$, 31.9% $^{18}O^{16}O$, and 63.0% $^{18}O_2$, and [^{18}O]acetate was 9.5% $^{16}O_2$, 38.9% $^{18}O^{16}O$, and 51.6% $^{18}O_2$. Protein A, 88 μg; protein B, 0.3–1 mg; and protein C, 189 μg. The reaction was incubated for 90–120 min at 30 °C in which 31–50% of glycine was deaminated. *p*-Phenylphenacyl acetate ^{18}O values for the reaction with glycine are the average of two enzyme reactions. Estimated error is ±3%. [b] Number of ^{18}O atoms in carboxyl group. [c] ≤1.4%; less than background values.

Scheme I: Proposed Mechanism of Action of Glycine Reductase



placing H_2O. The data also show that some glycine molecules, which originally contained two ^{18}O atoms, lost both ^{18}O atoms upon conversion to product. This indicates that formation of the acyl enzyme is reversible. However the acyl enzyme does not revert to free glycine since the remaining glycine has not lost ^{18}O. This point will be discussed further below in relation to a specific reaction sequence. These data are inconsistent with the formation of acetyl phosphate from enzyme-bound acetate and a phospho enzyme.

Conclusion

Glycine reductase catalyzes the formation of acetyl phosphate from glycine and inorganic phosphate. The previous observation that ATP and acetate are the products of the reaction is due to an acetate kinase contaminant. In addition, we have demonstrated that glycine labeled in the carboxyl group with ^{18}O loses ^{18}O upon conversion to product whereas the reisolated glycine has not lost ^{18}O.

A speculative mechanism for the action of glycine reductase is shown in Scheme I. As an initial step we propose the formation of a Schiff base (I) between glycine and the carbonyl cofactor of protein B. This makes the amino group a better leaving group and facilitates the next step in the sequence, the displacement of the nitrogen by an SH group at the active site (I → II). This displacement reaction may seem unattractive on chemical grounds. It should be remembered, however, that betaine–homocysteine methyltransferase (Awad et al., 1983) catalyzes a similar displacement reaction. In that reaction an SH group of homocysteine attacks a methyl group and displaces a tertiary amino group (dimethylglycine). As a leaving group the imine of I (Scheme I) is comparable to dimethylglycine. Next, an acyl enzyme is formed by reaction of the substrate carboxyl group and a nucleophile (X^-) (II → III). The equilibrium of this reaction is expected to be unfavorable, but reactions of this type are not without precedence. For instance, acetylcholinesterase catalyzes the exchange of ^{18}O from solvent H_2O into the carboxyl group of acetate (Bentley & Rittenberg, 1954). The reduction of the α-carbon is achieved by attack of a thiolate group on the thioether (III → IV). Again, this reaction has chemical precedence (Cram & Cordon, 1955). The resulting enol ketonizes to give the acyl enzyme (IV → V), which reacts with P_i to form acetyl phosphate. To be consistent with the oxygen-exchange data, intermediate III can reverse to II but not to form glycine. This is reasonable since breaking of the carbon–nitrogen bond would be expected to be an irreversible process. The latter part of this mechanism (acyl enzyme formation and phosphorolysis) is analogous to the mechanism of glyceraldehyde-3-phosphate dehydrogenase.

Acknowledgments

We thank Nyla Spawn for help in preparing the manuscript.

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