Mechanism of Action of Clostridial Glycine Reductase: Isolation and Characterization of a Covalent Acetyl Enzyme Intermediate†

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ABSTRACT: Clostridial glycine reductase consists of proteins A, B, and C and catalyzes the reaction glycine + P_i + $2e^-$ → acetyl phosphate + NH_4^+. Evidence was previously obtained that is consistent with the involvement of an acyl enzyme intermediate in this reaction. We now demonstrate that protein C catalyzes exchange of [^{32}P]P_i into acetyl phosphate, providing additional support for an acetyl enzyme intermediate on protein C. Furthermore, we have isolated acetyl protein C and shown that it is qualitatively catalytically competent. Acetyl protein C can be obtained through the forward reaction from protein C and *Se*-(carboxymethyl)selenocysteine–protein A, which is generated by the reaction of glycine with proteins A and B [Arkowitz, R. A., & Abeles, R. H. (1990) *J. Am. Chem. Soc. 112*, 870–872]. Acetyl protein C can also be generated through the reverse reaction by the addition of acetyl phosphate to protein C. Both procedures lead to the same acetyl enzyme. The acetyl enzyme reacts with P_i to give acetyl phosphate. When [^{14}C]acetyl protein C is denaturated with TCA and redissolved with urea, radioactivity remained associated with the protein. At pH 11.5 radioactivity was released with $t_{1/2}$ = 57 min, comparable to the hydrolysis rate of thioesters. Exposure of 4 N neutralized NH_2OH resulted in the complete release of radioactivity. Treatment with KBH_4 removes all the radioactivity associated with protein C, resulting in the formation of [^{14}C]ethanol. We conclude that a thiol group on protein C is acetylated. Proteins A and C together catalyze the exchange of tritium atoms from [^{3}H]H_2O into acetyl phosphate. This exchange reaction supports the proposal [Arkowitz, R. A., & Abeles, R. H. (1989) *Biochemistry 28*, 4639–4644] that an enol of the acetyl enzyme is an intermediate in the reaction sequence.

Amino acid fermenting anaerobic bacteria reductively deaminate diverse amino acids (Stickland, 1934; Stadtman, 1954; Naumann et al., 1983; Zindel et al., 1988; Hormann & Andreesen, 1989). For example glycine is converted to acetyl phosphate and NH_4^+ (Arkowitz & Abeles, 1989), and D-proline is converted to δ-aminovaleric acid (Stickland, 1935). Recently Naumann et al. (1983) and Hormann and Andreesen (1989) have shown that *Clostridium sporogenes* and *Eubacterium acidaminophilum* reductively deaminate betaine to trimethylamine and acetate, and sarcosine to methylamine and acetate. Growth studies suggest that selenite is required for both activities (Naumann et al., 1983; Zindel et al., 1988). Glycine and proline reductases contain covalently bound pyruvate (Arkowitz, 1990; Hodgins & Abeles, 1967), which appears to be involved in the catalytic process, since reduction with $NaBH_4$ or reaction with NH_2OH leads to loss of catalytic activity (Hodgins & Abeles, 1967; Tanaka & Stadtman, 1979). Betaine and sarcosine reduction in cell-free extracts was significantly less sensitive to NH_2OH treatment than the corresponding glycine reductase activity (Hormann & Andreesen, 1989), suggesting that these two enzyme systems do not possess a carbonyl cofactor.

Glycine and proline reductases are the most well characterized amino acid reductases, and these enzymes have been purified to near homogeneity from *Clostridium sticklandii* (Seto & Stadtman, 1976; Tanaka & Stadman, 1979). Glycine reductase catalyzes the reaction (Arkowitz & Abeles, 1989)

$$^+NH_3CH_2COO^- + P_i + 2e^- \rightarrow CH_3COOPO_3^{2-} + NH_4^+ \quad (1)$$

The clostridial glycine reductase is a complex consisting of three protein components: protein A and two membrane-associated proteins, B and C (Tanaka & Stadtman, 1979). Protein A contains a selenocysteine residue (Cone et al., 1976) and two cysteine residues, at least one of which is necessary for activity (Stadtman, 1966). Protein B contains a covalently bound pyruvate cofactor (Arkowitz, 1990). We are interested in the chemical mechanism of this reaction because of the unusual reaction that is catalyzed, i.e., the reductive cleavage of a carbon–nitrogen bond and concomitant formation of the high-energy compound acetyl phosphate. We have proposed the mechanism shown in Scheme I.

We also found that, in the presence of protein B, protein A reacts with glycine to form a covalent protein A–(carboxymethyl)selenocysteine intermediate (Arkowitz & Abeles, 1990). This established a catalytic role for the selenocysteine residue on protein A. SeCMSeCys–A[1] produces acetyl phosphate upon addition of protein C and P_i in accordance with the proposed mechanism. We have also shown that when glycine labeled with ^{18}O in the carboxyl group is converted to product, more than 1 atom of ^{18}O is lost (Arkowitz & Abeles, 1989). This observation provides evidence for the intermediate formation of an acetyl enzyme as proposed in the mechanism shown in Scheme I. Subsequently, Stadtman

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[1] Abbreviations: SeCMSeCys–A, Protein A–(carboxymethyl)selenocysteine intermediate (Scheme I, complex 2A); Ac-C_{rev}, acetyl enzyme derived from protein C and acetyl phosphate (Scheme I, complex 5C); Ac-C_{for}, acetyl enzyme generated from SeCMSeCys–A and protein C (Scheme I, complex 5C); glycine reductase, proteins A, B, and C; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid.

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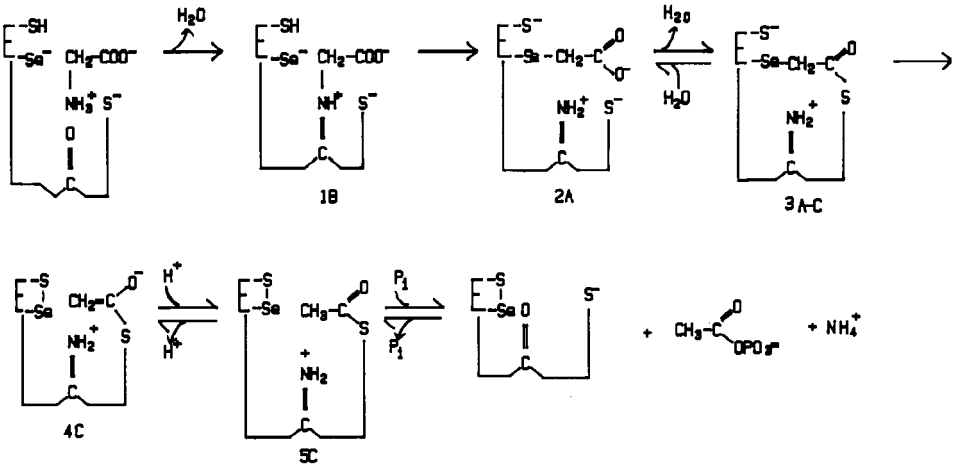
Scheme I: Proposed Mechanism of Action of Glycine Reductase[a]



[a] The letters indicate the protein involved in the formation of the complex. For instance, 1B indicates that complex 1 is formed with protein B.

(1989) demonstrated that protein C catalyzes the arsenolysis of acetyl phosphate, consistent with the involvement of an acetyl enzyme intermediate.

We now report the isolation and characterization of the acetyl enzyme.

Materials and Methods

Reagents. ATP (Na salt), acetyl phosphate (Li, K salt), coenzyme A (Li salt), alkaline phosphatase (bovine intestinal mucosa type I-S and VII-NT), phosphotransacetylase (*Clostridium kluyveri*), *S*-acetyl coenzyme A synthetase (bakers' yeast), malic dehydrogenase (bovine heart), citrate synthase (porcine heart), coenzyme A (Li salt), and DTNB were purchased from Sigma Chemical Co. DTT and Tricine were from Research Organics Inc. KBH_4 was purchased from Metal Hydrides Inc. Acetohydroxamate was purchased from Aldrich Chemical Co. and recrystallized from CH_3CN prior to use. Urea was purchased from J. T. Baker Inc. and was recrystallized, followed by purification with a mixed cation/anion Dowex exchange resin prior to use. Citrate was purchased from Fisher Scientific. [1,2-$^{14}C_2$]Glycine (100 mCi/mmol) was from ICN and was purified as described previously (Arkowitz & Abeles, 1989). [1-^{14}C]Glycine (52 mCi/mmol) from New England Nuclear was 99.9% radiochemically pure by cellulose TLC (system H). [^{3}H]CH_3CO-ONa (3300 mCi/mmol) and [1-^{14}C]CH_3COONa (58 mCi/mmol) were purchased from New England Nuclear. [^{32}P]-H_3PO_4 (5 mCi/mL, carrier-free) from New England Nuclear was 99% radiochemically pure by anion-exchange HPLC (system A).

Chromatographic Systems. The following were the conditions for each system: system A, Spherisorb SAX anion-exchange HPLC (4.6 × 250 mm, Alltech), gradient elution at 0.8 mL/min, solvent A 50 mM KH_2PO_4, solvent B 1 M KH_2PO_4, 5-min isocratic elution with 100% solvent A followed by a 10-min linear gradient to 40% solvent B, and thereafter isocratic elution was continued at 40% solvent B; system B, Bio-Rad Aminex ion-exclusion HPX-87H HPLC (7.8 × 300 mm), isocratic elution at 0.6 mL/min, solvent 5 mM H_2SO_4; system C, μBondpak C-18 reverse-phase HPLC (3.9 × 300 mm, Waters Associates), isocratic elution a 1 mL/min, solvent 10% MeOH in 20 mM ammonium acetate, pH 5.8; system D, Sephadex G-15 fine Penefsky column (Penefsky, 1979) in a 1-mL syringe, solvent 50 mM KH_2PO_4, centrifuged in clinical centrifuge for 2 min to remove protein, followed by an additional 2-min spin with 100 μL of solvent, eluted with 1 mL of solvent and spun for an additional 2 min; system E, Sephadex G-15 fine Penefsky column (Penefsky, 1979) in a 1-mL syringe, solvent 100 mM citrate, pH 6.0, at 4°, prespun to pack resin for 2 min in clinical centrifuge, eluted by 2-min spin; system F, Sephadex G-25 FPLC Pharmacia column (HR10/10), isocratic elution at 2 mL/min, solvent 100 mM Tricine/KOH, pH 7.5, and 1 mM DTT; system G, Spherogel TSK4000SW gel-filtration HPLC (7.5 × 300 mm, Beckman), isocratic elution at 0.5 mL/min, solvent 100 mM K_2HPO_4 pH 7.2, 1 mM DTT, and 1 mM MgK_2EDTA; system H, cellulose TLC sheets (Kodak), solvent system *t*-BuOH/acetic acid/H_2O (9:1:4).

Synthetic Procedures

Synthesis of [^{3}H]Acetyl Phosphate. A typical synthesis was done as follows: [^{3}H]CH_3COONa, 1.6 mCi (0.57 μmol), was brought to dryness under a stream of N_2. This residue was taken up in 5 mL of 1.7 mM CH_3COONa, 2.8 mM coenzyme A, 3.9 mM ATP, 4 mM $MgCl_2$, 100 mM Tris/HCl, pH 7.5, with 0.6 unit of *S*-acetyl coenzyme A synthetase (1 unit produces 1 μmol of AcCoA min^{-1} at pH 7.5, 37 °C added. The reaction mixture was incubated for 1 h at 37 °C and was quenched with 300 μL of 1 N HCl. After centrifugation, the supernatant fluid was loaded onto a Waters C-18 Sep-Pak cartridge equilibrated with 1 mM HCl. The cartridge was washed with 4 × 1 mL H_2O to remove unreacted [^{3}H]CH_3-COONa, and [^{3}H]acetyl-CoA was eluted with 50% MeOH in 1 mM HCl. MeOH was removed under a stream of N_2 followed by rotary evaporation under reduced pressure. The sample was finally concentrated to 860 μL by lyophilization. The yield based upon radioactivity was 42%.

The purified [^{3}H]acetyl-CoA (approximately 3.8 μmol) was added to a solution containing 0.8 mM K_2HPO_4, 20 mM $(NH_4)_2SO_4$, 1.2 mM DTNB, and 50 mM Tricine/KOH, pH 7.5, in a final volume of 5 mL. The reaction was started by the addition of 260 units of phosphotransacetylase and allowed to proceed at 37 °C for 1 h. The reaction was quenched by the addition of 50 mg of activated carbon (Darco G-60), clarified by centrifugation, and the supernatant fluid was loaded onto a Waters C-18 Sep-Pak cartridge equilibrated in H_2O. When necessary, the eluate was treated with activated carbon a second time, lyophilized to 430 μL, and frozen immediately in small aliquots. The yield of [^{3}H]acetyl phosphate from [^{3}H]acetyl-CoA based on total radioactivity was 72%. The radiochemical purity of the [^{3}H]acetyl phosphate was 86% with approximately 10% [^{3}H]acetate present, as determined by anion-exchange HPLC analysis (system A). Maximally, this material contained 0.7 mol of P_i/mol of acetyl phosphate.

[^{14}C]Acetyl phosphate was synthesized following the same procedure.

Synthesis of [^{32}P]Acetyl Phosphate. [^{32}P]Acetyl phosphate was synthesized following the procedure of Bodley and Jencks (1987), except that the reaction was scaled down 400-fold to 125 mM [^{32}P]KH_2PO_4 (1.3 mCi) in a reaction volume of 40 μL. In addition, the procedure was modified by adding acetic anhydride in DMF (1:1). The acetyl phosphate concentration was determined by a phosphotransacetylase, citrate synthase, and malic dehydrogenase coupled enzyme assay, measuring the initial velocity of NAD reduction at 340 nm (Bergmeyer & Moellering, 1974). The yield was 74%. The radiochemical purity of the [^{32}P]acetyl phosphate was 93% with approximately 7% [^{32}P]P_i present, as determined by anion-exchange HPLC analysis (system A).

Synthesis of Acetyl-CoA. Acetyl-CoA was synthesized from acetic anhydride following the procedure of Kuchta and Abeles (1985). The concentration of acetyl-CoA was determined by DTNB (Fendrich, 1983).

Assays

Glycine Reductase. The activity of reconstituted glycine reductase was assayed by measuring the amount of ^{14}C-labeled anionic product formed from [^{14}C]glycine as described previously (Arkowitz & Abeles, 1989). The standard assay mixture contained 60 mM Tricine/KOH, pH 8.1, 20 mM K_2HPO_4, 8 mM $MgCl_2$, 40 mM DTT, 40 mM [^{14}C]glycine (5000–6000 cpm/μmol), and 10 units of alkaline phosphatase in a final volume of 250 μL.

Protein C Arsenolysis Assay. In addition to assaying protein C by reconstitution with proteins A and B as described above, protein C was also assayed by the arsenolysis of acetyl phosphate. The reaction conditions were identical with those of the standard assay mixture except that alkaline phosphatase was omitted, 40 mM acetyl phosphate replaced the glycine, and 40 mM Na_2HAsO_4 replaced the K_2HPO_4. The reaction was started by the addition of DTT and was also carried out under an atmosphere of argon. After 30 min at 30 °C, the remaining acetyl phosphate was determined by the method of Lipmann and Tuttle (1945). Typically, 50 μL of the reaction mixture was added to 950 μL of H_2O and 1 mL of 2 M freshly neutralized NH_2OH·HCl. After 30 min at 25 °C, 4 mL 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.

Enzyme Isolation

Protein A. Protein A was purified from *C. sticklandii* cells by a modification of the procedure of Turner and Stadtman (1973) as described (Arkowitz & Abeles, 1989). When required, the Bio-Rad P-30 gel-filtration chromatography step was repeated after FPLC Pharmacia Mono Q anion-exchange chromatography. The specific activity of protein A in the presence of saturating amounts of proteins B and C was 85–90 μmol of anionic product (90 min)$^{-1}$ (mg of protein)$^{-1}$. Protein A was typically ≥95% pure as judged by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

Proteins B and C. Proteins B and C were resolved as described previously (Arkowitz & Abeles, 1989). Protein B was further purified by FPLC Mono Q anion-exchange chromatography as described (Arkowitz & Abeles, 1989). Alternately proteins B and C were separated by gel-filtration chromatography on a 3.4 × 112 cm Sepharose 6B-Cl column. The column was equilibrated and eluted as described previously for the Sephacryl S-300 column (Arkowitz & Abeles, 1989) except that the flow rate was 18 mL/h. Under these conditions, protein C eluted between 645 and 765 mL of eluent and protein B between 1000 and 1125 mL of eluent. Protein C was concentrated in an Amicon centriprep and stored at 4 °C. The specific activity of protein C in the presence of saturating amounts of proteins A and B was 50–70 μmol of anionic product (90 min)$^{-1}$ (mg of protein)$^{-1}$. Protein C migrated as one major band on native polyacrylamide gel electrophoresis (Davis, 1964) and as four major bands on SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Protein B from the Sepharose 6B-Cl column was further purified by Mono Q FPLC anion-exchange chromatograpy. The specific activity of protein B was 24–30 μmol of anionc product (90 min)$^{-1}$. This material was ≥25% pure as judged by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). In experiments in which [^{14}C]SeCMSeCys–A was generated, protein B from the Sepharose 6B-Cl column chromatography step was used.

Isolation of Acetyl Protein C

Isolation of [^{3}H]Ac-C_{rev} from [^{3}H]Acetyl Phosphate. Protein C was dialyzed versus 100 mM Tricine buffer pH 7.5, in order to remove $(NH_4)_2SO_4$ and P_i, immediately prior to use. Protein C (15.9 mg of protein) was added to 2.2 mM [^{3}H]acetyl phosphate (179 mCi/mmol) in a final volume of 500 μL. This mixture was incubated at 25 °C for 5 min, then chilled on ice, and loaded onto a 1 × 35 cm Bio-Rad P-30 gel-filtration column equilibrated in 100 mM Tricine, pH 7.5, at 4°. Fractions (1 mL) were collected at a flow rate of 0.3 mL/min. Aliquots (10 μL) of each fraction with 90 μL of 100 mM Tricine, pH 7.5, added were measured for ^{3}H radioactivity. Protein concentrations were determined by the method of Lowry (1951).

Reaction of [^{3}H]Ac-C_{rev} with P_i. [^{3}H]Ac-C_{rev} was isolated as described above. This intermediate (0.1 nmol of label; 3.9 mg of protein) was taken up in 23 mM inorganic phosphate and 1 mM acetyl phosphate carrier. The reaction was incubated at 25 °C for 10 min and then loaded onto a Waters C-18 Sep-Pak cartridge equilibrated in 50 mM KH_2PO_4 at 4 °C. The Sep-Pak was eluted with 5 × 1 mL 50 mM KH_2PO_4 at 4 °C, and the pooled radioactivity was injected onto an anion-exchange HPLC column (system A). The concentrations of acetyl phosphate in the eluted fractions were determined by the phosphotransacetylase, citrate synthase, and malic dehydrogenase coupled enzyme assay (Bergmeyer & Moellering, 1979).

TCA Precipitation of [^{14}C]Ac-C_{rev}. Protein C (3.7 mg of protein) was desalted on a FPLC Sephadex G-25 column (system F). An aliquot of this material (1.4 mg of protein) was incubated with 3 mM [^{14}C]acetyl phosphate (58 mCi/mmol) at 25 °C for 5 min. The reaction was chilled to 4 °C and subsequent procedures were carried out at this temperature. The reaction was then loaded onto a Sephadex G-15 Penefsky column (system E) and eluted into 0.5 mL of 5% TCA. The eluent was transferred to an equal volume of 5% TCA solution, and the protein precipitate was removed by centrifugation. The pellet was solubilized with 120 μL of 6 M urea and then precipitated by the addition of 880 μL of TCA to a final concentration of 5%. The protein precipitate was removed by centrifugation, the supernatant fluid was discarded, and the pellet was resolubilized with 6 M urea. This procedure was repeated. Aliquots (5 or 10 μL) of this denatured labeled protein C (0.8 nmol of label/0.79 mg of protein; 6.6 mg/mL protein in 6 M urea) were added to an equal volume of either H_2O, 1 M Na_2CO_3, pH 11.5, 1 N HCl, 4 N freshly neutralized NH_2OH, or KBH_4 and incubated at 25 °C for the specified times. Samples were quenched by being brought up to 500 μL with 5% TCA at 4 °C and filtered

through a 250-mm diameter Millipore glass fiber filter (AP20 02500), which was prewashed with 5% TCA. The filter was subsequently washed with 3 × 1 mL of 5% TCA and radioactivity was determined. The apparent first-order rate constant for hydrolysis of [^{14}C]Ac-C_{rev} at pH 11.5, 25 °C, was determined by measuring the amount of remaining radioactive material at 0, 10, 20, 30, and 45 min and after 4 N NH_2OH treatment.

Isolation of [^{14}C]*C-*C_{for}. [^{14}C]SeCMSeCys–A was isolated essentially as previously described (Arkowitz & Abeles, 1990) except that [^{14}C]SeCMSeCys–A was separated from protein B on a 1 × 35 cm Bio-Rad P-60 gel filtration column at 4 °C equilibrated in 100 mM citrate, pH 6.0. Fractions containing [^{14}C]SeCMSeCys–A were pooled, brought up to 1 mM DTT, and concentrated in an Amicon Centricon 3. This isolated [^{14}C]SeCMSeCys–A was stable for at least a week at 4 °C. Protein C (2.8 mg of protein) that had been desalted on an FPLC Sephadex G-25 column (system F) was incubated in 1 mM DTT for 5-10 min at 4 °C prior to addition of [^{14}C]-SeCMSeCys–A. All subsequent procedures were carried out at 4 °C unless otherwise noted. [^{14}C]SeCMSeCys–A (3.6 nmol of label, 0.44 mg of protein) was then added to the protein C mixture resulting in a final volume of 100 μL, 200 mM citrate, pH 6.0. The reaction was incubated for approximately 30 s and then transferred to 16 °C for another 30 s. The mixture was subsequently chilled on ice and loaded onto a 20 × 0.5 cm Bio-Rad P-60 gel-filtration column equilibrated in 100 mM citrate, pH 6.0, at 4 °C. Flow rate was 4–5 mL/h, and 0.22-mL fractions were collected. Aliquots of the fractions were taken up to 100 μL with H_2O, and ^{14}C radioactivity was determined. Protein C eluted between fractions 9 and 11, [^{14}C]SeCMSeCys–A between fractions 13 and 17, and small molecules between fractions 26 and 30.

Reaction of [^{14}C]*Ac-*C_{for} *with* P_i. Immediately after [^{14}C]Ac-C_{for} eluted from the P-60 gel filtration column, 0.7 nmol of labeled protein C (0.8 mg of protein) was taken up in 52 mM KH_2PO_4 and 3 mM acetyl phosphate to a final volume of 385 μL and incubated at 25 °C for 5 min. This mixture was subsequently frozen and later injected onto an anion-exchange HPLC column (system A), and the specific activity of the eluted acetyl phosphate was determined as described above. Alternately, the [^{14}C]Ac-C_{for} was isolated as described, and KH_2PO_4 and acetyl phosphate were added to 0.12 nmol of labeled Ac-C_{for} under identical conditions. After incubation at 25 °C for 5 min, the sample was deproteinized with an Amicon Centricon. The filter was washed with an additional 500 μL of H_2O. The filtrate (740 μL) was brought up to 50 mM Tricine, pH 8.1, and 2 μmol of CoASH and 200 units of phosphotransacetylase were added. The reaction was allowed to proceed for 1 h at 25 °C. The acetyl-CoA was purified on a Waters C-18 Sep-Pak cartridge (Arkowitz & Abeles, 1989) and analyzed on a C-18 reverse-phase HPLC column (system C).

TCA Precipitation of [^{14}C]*Ac-*C_{for}. [^{14}C]SeCMSeCys-A (1.7 nmol of label, 0.34 mg of protein) was added to 0.6 mg of protein C, which had been preincubated in 1 mM DTT at 4 °C as described above. The reaction mixture was brought to 16 °C for 30 s and then immediately loaded onto a G-15 Sephadex Penefsky column (system E) and eluted into 0.5 mL of 5% TCA. The eluent was transferred to an equal volume of 5% TCA, and the protein precipitate was removed by centrifugation. The pellet was solubilized in 120 μL of 6 M urea. This material contained both [^{14}C]SeCMSeCys-A and [^{14}C]Ac-C_{for}. Aliquots (10 μL) of this denatured protein mixture (0.08 nmol of label, 49 μg of protein) were added to an equal volume of either H_2O, 1 M Na_2CO_3, pH 11.5, 1 N HCl, or 4 N freshly neutralized NH_2OH and incubated at 25 °C for the specified times. The reactions were quenched with 5% TCA, and the remaining protein-associated radioactive material was determined by using glass fiber filters as described above. The apparent first-order rate constant for hydrolysis of [^{14}C]AC-C_{for} at pH 11.5, 25 °C, was determined by measuring the amount of radioactivity remaining at 10, 20, 30, 45, and 60 min and after 4 N NH_2OH treatment.

Table I: Acetyl Phosphate/$^{32}P_i$ Exchange[a]

enzyme component(s)	nmol exchanged min^{-1}
32 μg of C	77
64 μg of C	140
32 μg of C, B, and A	77
32 μg of C and B	71

[a] Standard assay conditions except that alkaline phosphatase was omitted, 40 mM acetyl phosphate replaced glycine, and 50 mM [^{32}P]-K_2HPO_4 (11 700 Cerenkov cpm/μmol) was present. Protein B (240 μg) and 220 μg of protein A were added as indicated. The reaction was started by the addition of DTT and carried out under an atmosphere of argon. After 60 min of incubation at 30 °C, an aliquot (100 μL) was removed and immediately loaded onto a 1-mL Sephadex G-15 Penefsky column (system D). Acetyl phosphate was eluted with 1 mL of 50 mM KH_2PO_4. Samples were frozen to minimize hydrolysis prior to anion-exchange HPLC (system A) analysis. Acetate and inorganic phosphate eluted after 7 min and acetyl phosphate after 11 min. Fractions (0.4 mL) were collected and radioactivity was determined by Cerenkov counting. With these incubation times, the exchange reaction was under initial velocity conditions.

Results and Discussion

$^{32}P_i$/Acetyl Phosphate Exchange. In order to provide further evidence for an acetyl enzyme intermediate, the exchange of [^{32}P]P_i into acetyl phosphate was investigated. When glycine reductase is incubated with [^{32}P]K_2HPO_4 and acetyl phosphate, [^{32}P]P_i is exchanged into the reisolated acetyl phosphate (Table I). Protein C is sufficient for this exchange reaction, and the rate of exchange is dependent on the amount of protein C added. This exchange reaction is consistent with the protein C catalyzed arsenolysis of acetyl phosphate shown by Stadtman (1989).

The initial rate[2] of this exchange reaction is 48 ± 4 nmol of phosphate exchanged min^{-1} (32 μg of protein C)$^{-1}$. The initial velocity for the reduction of glycine is 60 ± 10 nmol of glycine reduced min^{-1} (32 μg of protein C)$^{-1}$, as determined by extrapolating to saturating amounts of proteins A and B. It was not possible to completely saturate protein C with increasing amounts of proteins A and B, in agreement with the observations of Tanaka and Stadtman (1979). These data are consistent with the intermediate formation of an acetyl enzyme, and the similarity between the rates of the catalytic reaction and the exchange reaction suggests that there is a common rate-limiting step in both reactions. The existence of a slow step in the catalytic reaction after acetyl enzyme formation is also in agreement with the loss of more than one ^{18}O atom during the conversion of [^{18}O]glycine to product (Arkowitz & Abeles, 1989).

Isolation of Acetyl Enzyme by Reaction of Protein C with Acetyl Phosphate. The exchange experiments suggested that the reaction of acetyl phosphate with protein C leads to the formation of an acetyl enzyme (referred to as Ac-C_{rev}). Experiments were carried out to determine if Ac-C_{rev} could be isolated. Protein C was incubated with [^{3}H]acetyl phosphate

[2] This average exchange rate was determined by using the fractional attainment of isotopic equilibrium at 10, 20, and 30 min and the equation for exchange between two species (Boyer, 1959).

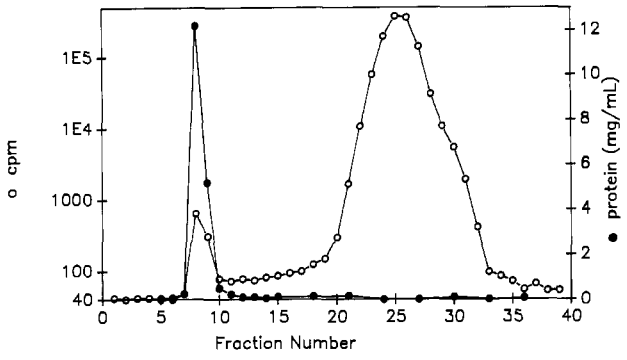


FIGURE 1: Isolation of [^{3}H]Ac-C_{rev} as described under Materials and Methods.

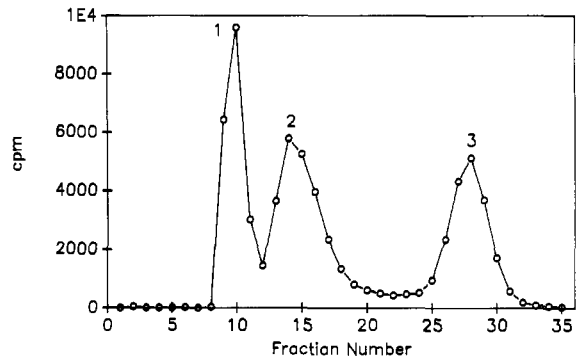


FIGURE 2: Isolation of [^{14}C]Ac-C_{for} as described under Materials and Methods. Peak 1, protein C; peak 2, protein A; and peak 3, small molecules.

under the conditions described under Materials and Methods for 5 min at 25 °C. This reaction mixture was subsequently chilled to 4 °C and loaded onto a gel-filtration column. Figure 1 shows that radioactive material (84 800 cpm) is associated with protein C. In a similar experiment, protein C (1.1 mg) purified by HPLC gel filtration (system G), to remove acetate kinase contaminants, was incubated with 2 mM [^{3}H,^{32}P]acetyl phosphate (140 mCi/mmol ^{3}H and 70 mCi/mmol ^{32}P; 1.5 ^{3}H cpm: $^{32}P_i$ Cerenkov cpm ratio), and labeled protein was isolated under identical conditions. The isolated protein C contained 17 400 ^{3}H cpm and 1400 ^{32}P Cerenkov cpm, ^{3}H:^{32}P = 12.4, whereas for the substrate acetyl phosphate ^{3}H:^{32}P = 1.5. The small amount of ^{32}P radioactivity associated with protein C is attributable to nonspecific binding of P_i or acetyl phosphate. These results demonstrate directly that an acetyl enzyme generated from acetyl phosphate can be isolated.[3]

Next, we determined whether Ac-C_{rev} can give rise to acetyl phosphate. Labeled Ac-C_{rev} was isolated as described above by using either [^{3}H]acetyl phosphate or [^{14}C]acetyl phosphate. Inorganic phosphate (23 mM) and unlabeled acetyl phosphate (1 mM) were added immediately to 0.1 nmol of Ac-C_{rev} (3.9 mg of protein). The mixture was incubated for 10 min at 25 °C, and the released radioactivity was analyzed with anion-exchange HPLC (system A). Between 30 and 60% (based upon the specific activity of the reisolated acetyl phosphate from three separate experiments) of the radioactive material originally associated with protein C comigrated with acetyl phosphate and the remainder cochromatographed with acetate.

Chemical Characterization of Ac-C_{rev}. The sensitivity of [^{14}C]Ac-C_{rev} to acidic and basic conditions, NH_2OH, and KBH_4 was examined in order to characterize the linkage of the acetyl group to protein C. [^{14}C]Ac-C_{rev} was prepared as described above except that the reaction mixture was passed through a Sephadex G-15 Penefsky column (system E) into 0.5 mL of 5% TCA. The precipitated protein was solubilized with 6 M urea. TCA precipitation followed by urea solubilization was repeated until no further radioactivity was detected in the supernatant fluid. Radioactivity (0.6–1.0 nmol of label/mg of protein)[3] was associated with the denatured protein C, and this radioactive material remained protein bound upon incubation at 25 °C for 5 min. When an aliquot of this labeled protein was incubated with freshly neutralized 4 N NH_2OH for 10 min at 25 °C, less than 1% of the radioactive material remained protein bound. All radioactive material remained protein bound after 9 h at 25 °C in 0.5 N HCl. Furthermore, when the labeled protein was incubated in 0.5 M Na_2CO_3, pH 11.5, at 25 °C, the apparent first-order rate constant for hydrolysis of the acetyl group was 1.2×10^{-2} min^{-1}, consistent with the rate of hydrolysis of thioesters (5×10^{-2} min^{-1}, for $CH_3COSCH_2CH_3$ and 4×10^{-2} min^{-1} for $CH_3COSCH_2CH_2CH_3$ at 35 °C, 10 mM NaOH) (Idoux et al., 1973). The complex was also treated with KBH_4. [^{14}C]Ac-C_{rev} (0.1 nmol of label) in 6 M urea with unlabeled acetate and ethanol added was allowed to react with KBH_4 for 10 min at 25 °C. The reaction mixture was subsequently quenched with $HClO_4$. All of the released radioactivity (85% based upon specific activity of the eluted ethanol) cochromatographed with ethanol on ion-exclusion HPLC (system B). The stability of Ac-C_{rev} under acidic and basic conditions and its sensitivity to KBH_4 reduction indicate that the acetyl group is attached to protein C through a thioester linkage.

Isolation of Acetyl Enzyme Derived from Glycine. Up to this point acetyl enzyme was generated from acetyl phosphate. The mechanism requires that it should also be possible to form this intermediate from glycine. Experiments were carried out to determine if such an intermediate (referred to as Ac-C_{for}) could be isolated from glycine in a stepwise fashion. [^{14}C]-SeCMSeCys–A (3.6 nmol), isolated as described previously (Arkowitz & Abeles, 1990), was added to protein C (2.8 mg) as described under Materials and Methods. The mixture was then loaded onto a gel-filtration column that separates proteins C and A. Figure 2 shows that three radioactive peaks were detected, corresponding to protein C, protein A, and acetate. One nanomole of radioactive material (28% of the total radioactivity) eluted with protein C ([^{14}C]Ac-C_{for}). In addition, 1.2 nmol of label (33% of the total radioactivity) eluted with protein A, as SeCMSeCys–A, and 1 nmol of radioactive material eluted as acetate. When the experiment described in Figure 2 was repeated, except that [^{14}C]SeCMSeCys–A and protein C were allowed to react for 5 min at 25 °C, >90% of the radioactive material was found in the small molecule peak (acetate). Without protein C present, the radioactivity associated with [^{14}C]SeCMSeCys–A remains protein bound. Furthermore, when [^{14}C]SeCMSeCys–A was alkylated with ICH_2COONa (Arkowitz, 1990), excess alkylating agent was removed, and then protein C was added as described in Figure 2, no radioactivity was transferred to protein C. These results show that the acetyl moiety of SeCMSeCys–A can be transferred to protein C, generating acetyl protein C (Ac-C_{for}), and suggest that the cysteine residues of SeCMSeCys–A are necessary for this transfer.

It is important to demonstrate that Ac-C_{for}, isolated as described above, and Ac-C_{rev}, isolated from protein C and

[3] The number of actives sites in native protein C is unknown. If one assumes a native molecular weight of 400 000 (Stadtman, 1989) and a purity of 50%, then the stoichiometry observed for Ac-C_{rev} isolated by gel filtration ranged between 0.04 and 0.16 mol of acetyl group/mol of protein. This apparent low stoichiometry is attributable to the instability of native Ac-C_{rev}. The stoichiometry of Ac-C_{rev} isolated immediately after formation by TCA precipitation ranged from 0.5 to 0.8.

acetyl phosphate, are the same. Initially, we examined the ability of Ac-C_{for} to form acetyl phosphate from inorganic phosphate. [^{14}C]Ac-C_{for} (0.7 nmol) was incubated with 52 mM KH_2PO_4 and 3 mM acetyl phosphate for 5 min at 25 °C. When the released radioactive material was analyzed as described earlier, 57% of the radioactivity associated with protein C formed acetyl phosphate and the remainder comigrated with acetate. In another experiment, acetyl phosphate released from [^{14}C]Ac-C_{for} was trapped as acetyl-CoA with phosphotransacetylase and CoASH. Sixty-six percent of the radioactivity originally associated with Ac-C_{for} cochromatographed with acetyl-CoA on C-18 reverse-phase HPLC (system C). These experiments establish that the acetyl enzyme derived from glycine (Ac-C_{for}) gives rise to acetyl phosphate in the presence of P_i, as dose Ac-C_{rev}.

Chemical Characterization of Ac-C_{for}. In order to provide further evidence that Ac-C_{for} is similar to Ac-C_{rev}, we examined the stability of Ac-C_{for} under various conditions. KBH_4 was added to native [^{14}C]Ac-C_{for} isolated by P-60 gel-filtration chromatography in 100 mM citrate, pH 6.0, and the released radioactivity was analyzed on an Aminex ion-exclusion HPLC column (system B). Fifty-four percent (average of three experiments) of the radioactivity initially associated with protein C comigrated with ethanol and the remaining radioactivity cochromatographed with acetate. In contrast, treatment of the [^{14}C]SeCMSeCys–A alone with KBH_4 resulted in only 3% [^{14}C]ethanol formation, consistent with previous work (Arkowitz & Abeles, 1990). KBH_4 reduces the same amount of Ac-C_{for} (54%) as that which reacts with P_i to form acetyl phosphate (57–66%). We examined the stability of Ac-C_{for} under acidic and basic conditions, using denatured Ac-C_{for}. [^{14}C]SeCMSeCys–A was added to protein C, the reaction was brought to 16 °C for 30 s, and then the sample was immediately loaded onto a Sephadex G-15 Penefsky column (system E) and centrifuged for 2 min into 0.5 mL of 5% TCA. The precipitated protein (which included both [^{14}C]SeCMSeCys–A and [^{14}C]Ac-C_{for}) was solubilized with 6 M urea. Approximately 40% of the protein-associated radioactive material was released upon incubation with freshly neutralized 4 N NH_2OH for 10 min at 25 °C. Therefore, 40% of the [^{14}C]SeCMSeCys–A has been transferred to protein C ([^{14}C]SeCMSeCys–A is stable to NH_2OH treatment). The apparent first-order rate constant for hydrolysis of the acetyl group of the denatured Ac-C_{for} at 25 °C, in 0.5 M Na_2CO_3, pH 11.5, was 1.1×10^{-2} min^{-1}, in good agreement with the value for Ac-C_{rev}. Furthermore, greater than 90% of the label remained associated with the protein after incubation for 3 h at 25 °C in 0.5 N HCl. The pH stability and KBH_4 reactivity of Ac-C_{for} indicates that the acetyl moiety is transferred to a cysteine residue on protein C, forming a thioester. These results taken together indicate that Ac-C_{for} is identical with Ac-C_{rev}.

The experiments cited above establish the existence of an intermediate acetyl enzyme occurring on protein C. We considered the possibility that the carboxyl group of SeCMSeCys–A forms an ester linkage to protein A. To address this possibility, we treated [^{14}C]SeCMSeCys–A with either NH_2OH or KBH_4. [^{14}C]SeCMSeCys–A was generated as described previously (Arkowitz & Abeles, 1990). Prior to separation of proteins A, B, and [^{14}C]glycine by gel filtration, the reaction mixture was treated with either freshly neutralized 2 N NH_2OH or 1 M KBH_4. In either case, [^{14}C]SeCMSeCys–A was isolated and product was formed upon addition of protein C to this intermediate. These results rule out the possibility that the carboxylate of SeCMSeCys–A is esterified and accessible to modification.

Table II: Exchange of Tritium from [^{3}H]H_2O into Acetyl Phosphate[a]

enzyme component(s)	nmol exchanged h^{-1}
A, B, and C	75
A and C	52
C	
A	
2× (A and C)	156

[a] Standard assay conditions with 20 mM acetyl phosphate containing 2 mM inorganic phosphate and 1.6 mCi of [^{3}H]H_2O. Proteins A (70 μg), B (820 μg), and C (140 μg) were added as indicated. The reactions were incubated for 90 min at 25 °C and quenched with 10 units of alkaline phosphatase, and the acetate was purified by Dowex1(Cl^-) chromatography followed by analysis using ion-exclusion HPLC (system B). Specific activities of the eluted acetate were determined by using the integrations of the refractive index signals and ^{3}H radioactivity determinations. To ascertain that the radioactive material which eluted at 18 min was acetate and not residual [^{3}H]H_2O, this material was pooled, neutralized, and converted to the hydroxamate with acetate kinase in the presence of inorganic phosphate and neutralized NH_2OH. The hydroxamic acid was then analyzed by ion-exclusion HPLC (system B).

[^{3}H]H_2O Tritium Exchange into Acetyl Phosphate. According to the mechanism of Scheme I, glycine reductase could catalyze the exchange of the protons of acetyl phosphate with solvent tritons. This exchange could occur if complex 5C (Scheme I) can revert to complex 4C. Minimally, protein C should be necessary for such an exchange reaction. When glycine reductase was incubated for 90 min at 25 °C under standard assay conditions with acetyl phosphate in [^{3}H]H_2O, solvent tritium was incorporated into acetyl phosphate (Table II). In contrast to the P_i/acetyl phosphate exchange reaction, protein C alone was not sufficient for tritium exchange. Tritium exchange was observed only in the presence of both protein C and protein A, and neither protein A nor protein C alone catalyzed this reaction. It is not clear what the role of protein A is in the tritium exchange reaction, yet the forward catalytic reaction, the reductive deamination of glycine, is irreversible. The amount of tritium exchange was dependent on the incubation time and the amount of proteins A and C added, indicating that this reaction was under initial velocity conditions (Table II). Together proteins A and C catalyzed tritium exchange (not corrected for isotope effect) at an apparent rate of 56 nmol of acetyl phosphate exchanged h^{-1} (94 μg of A and 280 μg of C)$^{-1}$ in the presence of 2 mM P_i and 400 mM Na_2SO_4. Under identical conditions the rate of arsenolysis of acetyl phosphate was approximately 78 μmol of acetyl phosphate hydrolyzed h^{-1} (94 μg of A and 280 μg of C)$^{-1}$, 1400 times faster than tritium exchange. The tritium exchange reaction is slow compared to the forward catalytic reaction, presumably because the exchange reaction involves the deprotonation of Ac-C (the conversion of 5C to 4C), which may be slow. The slow rate of tritium exchange is consistent with the conclusion of Barnard and Akhtar (1979) that the protons of glycine are retained upon conversion to product.

According to Scheme I, Ac-C can either react with P_i to yield acetyl phosphate or revert to complex 4C. Conversion of 4C and 5C results in ^{3}H incorporation into acetyl phosphate. The rate of tritium exchange should be dependent on [P_i]. The rate should increase with increasing inorganic phosphate concentration. At high inorganic phosphate concentration, the rate of tritium exchange should decrease since the rate of interconversion of 5C to 4C would be reduced due to trapping of 5C by P_i. Figure 3 shows that the amount of ^{3}H exchange increases as inorganic phosphate concentration increases to 50 mM and subsequently decreases as P_i concentration is increased further. In addition, extremely little exchange (2

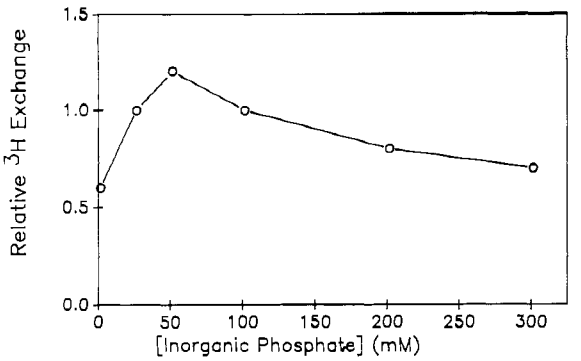


FIGURE 3: Tritium exchange from $[^3H]H_2O$ into acetyl phosphate. Reaction conditions were as described in Table II except ionic strength was maintained with Na_2SO_4 ($[K_2HPO_4]$ plus $[Na_2SO_4]$ equal to 400 mM). Protein A (94 μg) and protein C (280–440 μg) were incubated for 45 min at 25 °C. The reactions were quenched with 120 units of alkaline phosphatase and assayed for acetate as described in Table II.

nmol of acetyl phosphate exchanged h^{-1}) was observed when P_i was replaced by 40 mM Na_2HAsO_4, consistent with the above results. This partial exchange reaction is further evidence for intermediate carbanion formation.

The proposed mechanism of action of glycine reductase (Scheme I) involves covalent intermediates (1B, 2A, 3A-C, 4C, and 5C). Intermediate 2A had previously been isolated (Arkowitz & Abeles, 1990). In this paper we report the isolation of 5C and describe its properties. We have also obtained evidence for intermediate 4C. Furthermore, we have demonstrated qualitatively that all the isolated intermediates are catalytically competent.

Several aspects regarding the chemical steps involved in this mechanism deserve comment. We have proposed that the amino group of glycine forms an adduct, carbinolamine or imine, with the pyruvyl moiety of protein B, thereby enhancing its leaving ability. We have been unable to reductively trap such an adduct (Arkowitz, 1990). However, protein B is required to form complex 2A, and specifically the pyruvyl moiety of this protein is required for this reaction (Arkowitz & Abeles, 1990). In the formation of intermediate 2A, the selenoether protein A adduct, a displacement of the amino (or imino) group of glycine by the selenolate of SeCys is proposed. The displacement of $-NH_2$ is a difficult reaction. This displacement may be possible because the selenolate is a potent nucleophile (Pleasants et al., 1989; Huber & Criddle, 1967). For example benzeneselenol displaces cyclohexylamine from *N*-methylcyclohexylamine, resulting in (methylseleno)benzene (Reich & Cohen, 1979).

Generation of intermediate 3A-C involves the formation of a thioester linkage between the glycyl carboxyl group and a thiol on protein C. This reaction is probably facilitated by covalent and noncovalent binding interactions between substrate and enzyme that stabilize the thioester. Ample precedents exist for analogous reactions. For instance, chymotrypsin (Spinson & Rittenberg, 1951; Doherty & Vaslow, 1952) and acetylcholinestrase (Bentley & Rittenberg, 1954) catalyze the incorporation of solvent ^{18}O into the carboxyl group of carboxylic acids, presummably via an ester intermediate.

The actual reduction in the reaction catalyzed by glycine reductase occurs in the conversion of complex 3A-C to 4C. It is proposed that one of the sulfhydryl groups of protein A or possibly protein C attacks the selenium atom of the selenoether, resulting in the displacement of electrons toward the carbonyl oxygen and the cleavage of the carbon–selenium bond. Analogous intermolecular reactions for thioethers (Oki et al., 1971; Seshadri et al., 1981) and for selenoethers (Seshadri et al., 1981) have been reported.

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Evidence for the Development of an Intermonomeric Asymmetry in the Covalent Binding of 4,4′-Diisothiocyanatostilbene-2,2′-disulfonate to Human Erythrocyte Band 3†

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ABSTRACT: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) studies have identified two oligomeric forms of band 3 whose proportions on gel profiles were modulated by the particular ligand occupying the intramonomeric stilbenedisulfonate site during *intermonomeric* cross-linking by BS^3 [bis(sulfosuccinimidyl) suberate] [Salhany et al. (1990) *J. Biol. Chem. 265*, 17688–17693]. When DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) was irreversibly attached to all monomers, BS^3 covalent dimers predominated, while with DNDS (4,4′-dinitrostilbene-2,2′-disulfonate) present to protect the intramonomeric stilbenedisulfonate site from attack by BS^3, a partially cross-linked band 3 tetramer was observed. In the present study, we investigate the structure of the protected stilbenedisulfonate site within the tetrameric complex by measuring the ability of patent monomers to react irreversibly with DIDS. Our results show two main populations of band 3 monomers present after reaction with DNDS/BS^3: (a) *inactive* monomers resulting from the displacement of reversibly bound DNDS molecules and subsequent irreversible attachment of BS^3 to the intramonomeric stilbenedisulfonate site and (b) residual, *active* monomers. All of the residual activity was fully inhibitable by DIDS under conditions of reversible binding, confirming expectations that all of the monomers responsible for the residual activity have patent stilbenedisulfonate sites. However, within this active population, two subpopulations could be identified: (1) monomers which were irreversibly *reactive* toward DIDS and (2) monomers which were *refractory* toward irreversible binding of DIDS at pH 6.9, despite being capable of binding DIDS reversibly. Increasing the pH to 9.5 during treatment of DNDS/BS^3-modified cells with 300 μM DIDS did not cause increased irreversible transport inhibition relative to that seen for cells treated at pH 6.9. This result suggests that both well-defined DIDS-reactive lysine residues of the stilbenedisulfonate site have become unreactive toward DIDS within the refractory subpopulation of monomers. We suggest that the tetrameric structure generated by DNDS/BS^3 treatment is a unique quaternary state of band 3 characterized by a conformational asymmetry between active monomers at the stilbenedisulfonate binding site. In contrast, an alternate quaternary state is formed when all monomers are irreversibly bound by DIDS. This latter state yields covalent dimers on SDS–PAGE after intermonomeric cross-linking by BS^3. The significance of these two quaternary states to either the anion-exchange or the ankyrin binding functions of the porter is presently unknown.

Band 3 is the anion-exchange protein of the human erythrocyte membrane; it also binds cytosolic and cytoskeletal proteins (Passow, 1986; Salhany, 1990). We have recently identified two oligomeric forms of this porter based on in situ cross-linking with bis(sulfosuccinimidyl) suberate (BS^3)[1] (Salhany & Sloan, 1988, 1989; Salhany et al., 1990). One form was readily denatured in SDS under reducing conditions at room temperature to yield covalent[2] dimers. The other form was SDS-resistant up to 60 °C, yielding a noncovalent tetrameric complex composed of two covalent dimers. The population of the two forms was dependent on which ligand was bound to the intramonomeric stilbenedisulfonate site of band 3 during BS^3 intermonomeric cross-linking. Such structurally similar ligands as DIDS and DNDS yielded different cross-linked forms. We suggested that the two oligomeric forms might reflect different conformational states: one state (dimers) being stabilized consequent to the intro-

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[1] Abbreviations: DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; BS^3, bis(sulfosuccinimidyl) suberate; PLP, pyridoxal 5′-phosphate; Bistris, *N*,*N*-[bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FTI, fractional transport inhibition.

[2] In this paper, the term covalent means both covalent and irreversible when applied to the reactions of DIDS and BS^3 with band 3. The term reversible means the ionic binding of DIDS or BS^3 prior to the irreversible covalent reaction with band 3 lysines at the binding site. The term "DIDS-refractory" means unable to bind DIDS irreversibly but able to bind DIDS reversibly.

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