Riou, G., & Saucier, J. M. (1979) *J. Cell Biol. 82*, 248–263.
Riou, G., Belnat, P., & Bénard, J. (1980) *J. Biol. Chem. 255*, 5141–5144.
Saucier, J. M., Festy, B., & Le Pecq, J. B. (1971) *Biochimie 53*, 973–980.
Simpson, L. (1972) *Int. Rev. Cytol. 32*, 139–207.
Simpson, L., & Simpson, A. M. (1978) *Cell 14*, 169–178.
Smith, C. A., Jordan, J. M., & Vinograd, J. (1971) *J. Mol. Biol. 59*, 255–272.
Steinert, M. (1971) *Adv. Cytopharmacol. 1*, 229–240.
Wang, J. C. (1969) *J. Mol. Biol. 43*, 263–272.
Wang, J. C. (1974) *J. Mol. Biol. 89*, 783–801.
Watkins, T. I., & Woolfe, G. (1956) *Nature (London) 178*, 368–369.

Escherichia coli Photoreactivating Enzyme: Purification and Properties†

Robert M. Snapka‡ and Betsy M. Sutherland*

ABSTRACT: We have purified large quantities of *Escherichia coli* photoreactivating enzyme (EC 4.1.99.3) to apparent homogeneity and have studied its physical and chemical properties. The enzyme has a molecular weight of 36 800 and a $s^0_{20,w}$ of 3.72 S. Amino acid analysis revealed an apparent absence of tryptophan, a low content of aromatic residues, and the presence of no unusual amino acids. The N terminus is arginine. The purified enzyme contained up to 13% carbohydrate by weight. The carbohydrate was composed of mannose, galactose, glucose, and *N*-acetylglucosamine. The enzyme is also associated with RNA (approximately 10 nucleotides/enzyme molecule) containing uracil, adenine, guanine, and cytosine with no unusual bases detected.

Ultraviolet light (220–300 nm) produces cyclobutylpyrimidine dimers between adjacent pyrimidines on the same DNA strand (Setlow, 1963). These dimers have been shown to be a major cause of death and mutation in procaryotes and in a simple eucaryote and have been implicated in many effects of ultraviolet radiation on higher organisms (Setlow, 1973; Cleaver, 1968; Setlow et al., 1969; Sutherland et al., 1970; Hart et al., 1977). The DNA photoreactivating enzyme (EC 4.1.99.3) repairs UV[1]-irradiated DNA in a two-step reaction. The enzyme binds to the DNA (Rupert, 1962), presumably at the dimer, forming a metastable complex. On absorption of a photon in the range 300–600 nm, the enzyme catalyzes the photolysis of the dimer (Setlow et al., 1965), thus producing two monomer pyrimidines and restoring biological integrity to the DNA.

Although the enzyme is important because of its role in DNA repair and as an enzyme requiring light for catalysis, fundamental characterization of the enzyme has been impeded by several problems. First, there are only 10–20 photoreactivating enzyme molecules per *Escherichia coli* cell (Harm, 1964; Kondo & Kato, 1966). Second, the enzyme is labile, frequently losing activity, especially during ion-exchange chromatography. Third, the enzyme aggregates in solution, making difficult the determination of an accurate molecular weight (Sutherland et al., 1973). Finally, the enzyme has a low affinity for commonly used protein stains, making its localization on gels difficult; furthermore, the low absorbance of the apoprotein at 280 nm hampers its detection by absorbance measurements at that wavelength.

We have used an *E. coli* K-12 strain with greatly increased photoreactivating enzyme levels (Sutherland et al., 1972) as a source of large quantities of enzyme. We have developed a gentle purification procedure which allows the production of large quantities of active enzyme. Using methods designed to minimize aggregation and localizing the enzyme by its associated nucleotide-containing "cofactor", we have determined the molecular weight of the native enzyme to be 36 800 and that of the apoprotein to be 35 200 ± 200. We have also characterized the amino acid and carbohydrate composition of the enzyme. The apparent absence of tryptophan makes unlikely dimer photoreactivation by the enzyme by tryptophan photocatalysis.

Experimental Section

Assay for Photoreactivation. Samples were assayed for dimer photoreactivation by the method of Sutherland & Chamberlin (1973). All assays were carried out by using yellow Sylvania gold lamps as safelights.

Binding Assay. Fractions were tested for their ability to bind to ultraviolet (UV)-irradiated DNA by a filter binding assay, similar to that of Madden et al. (1973). Samples were added to 0.2 mL of an assay mix consisting of 20 mM KPO_4 buffer, pH 7.2, 62.5 mM NaCl, 0.1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2.5% dimethyl sulfoxide (Me_2SO), 0.25 mM calf thymus DNA, and 30–100 pmol of UV-irradiated ^{32}P-labeled T7 DNA. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 1.0 mL of an ice-cold rinse buffer (20 mM KPO_4 buffer, pH 7.2, 62.5 mM NaCl, 0.1 mM DTT, and 1.0 mM EDTA). Samples were then filtered through

† From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973, and the Department of Molecular Biology, University of California, Irvine, California 92717. *Received November 5, 1979; revised manuscript received May 15, 1980.* This research was supported by Grant No. CA23096 from the National Cancer Institute, a Research Career Development Award (CA00466) to B.M.S., and the Department of Energy.

‡ Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations used: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; i.d., inner diameter; PRE, photoreactivating enzyme; UV, ultraviolet.

0006-2960/80/0419-4201$01.00/0

Schleicher and Schuell cellulose acetate filters which had been presoaked with the rinse buffer. Filters were dried and counted on a Nuclear-Chicago planchet counter with a Geiger tube or in a LKB scintillation spectrometer.

Enzyme Purification. Photoreactivating enzyme was prepared from *E. coli* W3350 (λC1857S7 dg D ΔJ) (Sutherland et al., 1972). The first three steps of Sutherland et al. (1973) were followed; in brief, cells were disrupted by processing with glass beads in buffer E (10 mM Tris, pH 7.0, 0.1 mM DTT, and 0.1 mM EDTA) in a Waring Blendor. The beads were allowed to settle, and the supernatant liquid was centrifuged at 10000 rpm for 20 min in the SS34 rotor of the Sorval RCB. The supernatant fraction (fraction I) was adjusted to a protein concentration of 15 mg/mL, and 10% (w/v) streptomycin sulfate was added to a final concentration of 1%. The mixture was stirred for 15 min and centrifuged at 15000 rpm for 15 min. The pellet was resuspended in buffer E to the same volume as fraction I (after adjustment of protein concentration), and 5 M NaCl was added to a final concentration of 1 M. After 15 min, 10% (w/v) streptomycin sulfate was added to a final concentration of 1%; the mixture was stirred for 15 min and centrifuged as before. Solid ammonium sulfate (0.163 g/mL of fraction II) was added to the supernatant solution (fraction II); after ammonium sulfate had dissolved, the mixture was stirred for 15 min and centrifuged as before. An additional quantity of solid ammonium sulfate (0.163 g/mL of solution) was added, and the mixture was stirred and centrifuged as before. The pellets (fraction III), which contained over 90% of the enzyme activity, were resuspended in buffer E; glycerol was added to a final concentration of 40%, and the enzyme was stored at −20 °C. Fraction III was desalted by chromatography on a 2.5 × 20 cm column of Bio-Gel P-4 in buffer E. [For some experiments, 5 mg/mL pancreatic ribonuclease (heat treated at 90 °C for 10 min) was included in the buffer E used for resolubilization of fraction III. In this case, desalting was carried out on a 2.5 × 50 cm column of Bio-Gel P-30 in buffer E.] Desalting was followed by isoelectric focusing. The sample (10–20 mg) was loaded as part of the heavy solution in a 0–70% glycerol gradient with 3% ampholines (pH 3.5–10 or pH 4.0–6.0). An LKB 110-mL column was routinely used, and focusing was carried out at 600 V for at least 72 h at 4 °C. Two-milliliter fractions were collected, and pH measurements were made with a glass electrode immediately after elution of the column. Absorbances at 260 and 225 nm were monitored with a Zeiss Model PMQII spectrophotometer. The fractions were assayed for binding to UV-irradiated DNA, and those fractions with binding ability were assayed for photoreactivating activity. The pooled active fractions were designated fraction IV. This fraction contained aggregates of enzyme which could be solubilized by changing the pH from the isoelectric pH or by increasing the ionic strength (see below). Both soluble (fraction IV soluble) and precipitated (fraction IV precipitated) aggregates contained PRE activity. Ampholines were removed by chromatography on a 2.5 × 50 cm column of Bio-Gel P-30 in buffer E to yield fraction V.

Protein Assays. Protein concentrations were routinely determined by either a biuret assay (Layne, 1957) scaled down to 0.3-mL total volume or a fluorescamine assay (Udenfriend et al., 1972; Bohlen et al., 1973). Bovine serum albumin was used as a standard in both assays ($\epsilon_{1cm}^{1\%} = 6.6$ at 280 nm).

Amino Acid Analysis. Hydrolysis was carried out in 6 N HCl with 2% phenol and 4% thioglycolic acid at 110 °C under vacuum in sealed borosilicate tubes. Hydrolysates were analyzed on a Beckman Model 120C amino acid analyzer according to Spackman et al. (1958). For each analysis duplicate samples were hydrolyzed for 24, 48, and 72 h. Values for serine and threonine were extrapolated to zero time of hydrolysis.

Tryptophan Determination. Fraction IV enzyme was chromatographed on a 27 × 3 cm diameter column of Bio-Gel P-60 in 50 mM KPO_4, pH 7.2, 0.1 mM DTT, and 0.1 mM EDTA. The protein concentration was determined by a biuret assay. The enzyme was then examined by two methods for tryptophan content. First, the magnetic circular dichroic spectra (Barth et al., 1971; Holmquist & Vallee, 1978) were determined in a spectrometer described by Sutherland et al. (1976). The magnetic circular dichroism was also determined for RNA solutions (of similar concentration as the photoreactivating enzyme samples) to which tryptophan at known concentrations was added. These tests indicated that 1×10^{-6} M Trp could be detected in this spectrometer. Second, fluorescence excitation and emission spectra (in a Jobin-Yvon JY3 spectrofluorometer: 2-nm excitation slits, 4-nm emission slits) were determined for the intact enzyme and for the enzyme digested with α-chymotrypsin and Pronase and then diluted into 6 M urea by the method of Sasaki et al. (1975). Absorbances were kept below 0.3 by dilution or by use of 0.4-cm path (excitation)–1-cm path (emission) cuvettes to minimize internal absorption effects. Similar treatment of Trp solutions indicated that 1×10^{-6} M Trp could be detected in this manner.

$NaDodSO_4$ Gel Electrophoresis. The molecular weight of the enzyme was determined by using dansylated standards and unknowns (Inouye, 1971) and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (Shapiro et al., 1967). The enzyme was prepared for derivatization by heating the proteins to 60 °C for 10 min, centrifugation at 10000 rpm in a Sorvall SS34 rotor for 1 h, exhaustive dialysis against double-distilled water, and lyophilization to dryness. Reduction and carboxymethylation were carried out according to Weber & Osborn (1975). Gels (125 × 5 mm) were 5% acrylamide monomer and 0.13% bis(acrylamide). The running buffer was 0.1 M KPO_4, pH 7.1, 0.1% $NaDodSO_4$, and 10% glycerol. Electrophoresis was carried out at 10 mA/gel until the bromphenol blue tracking dye was within 5 mm of the end. Temperature was maintained by circulating room temperature water.

Anthrone Reaction. The anthrone reaction was that described by Mokrash (1954) scaled down to a 0.35-mL reaction volume.

Gas–Liquid Chromatography. Methanolysis of samples (225–350 μg of protein) was carried out in 1.2 N HCl/methanol for 12 h at 90 °C. Samples were then neutralized, treated for 12 h with acetic anhydride (0.15 mL), and dried over KOH in vacuo for 2 days. Sigma-Sil-A (0.05 mL) was added to the dry sample and reacted for 20 min at room temperature and 4 min at 85 °C. The silating agent was removed with a stream of dry N_2 and the sample taken up in CS_2 (50 μL). All samples contained arabitol (1–5 μg, about one-third of the anthrone-positive material of the sample) as a standard. Two to three microliters of derivatized sample was injected into a Hewlet-Packard 5930A gas chromatograph equipped with a 6 ft × 2 mm (inner dimension) column packed with 3% SE-30 on Supercoport, 100–200 mesh. The carrier gas was N_2 with a flow rate of 20 mL/min. The injector and detector were each kept at 260 °C, and the column temperature was programmed from 120 to 260 °C at a rate of 1 °C/min.

Analytical Ultracentrifugation. Samples were prepared by exhaustive dialysis against centrifuge buffer (10 mM tri-

ethanolamine hydrochloride, pH 7.5, 100 mM NaCl, 1.0 mM EDTA, and 0.05% β-mercaptoethanol) or buffer exchange in a molecular sieving column (Bio-Gel P-30, 50–100 mesh). Experiments were performed with the Beckman Spinco Model E analytical ultracentrifuge equipped with a RTIC temperature control system and UV-scanning optics. The partial specific volume of *E. coli* PRE was calculated as 0.74 mL/g from the amino acid composition. The density and viscosity of the centrifuge buffer were directly determined by measurements with a 10-mL Sprengel pycnometer and a Ostwald viscometer, respectively.

Molecular Sieve Chromatography in Guanidinium Chloride. The molecular weights of unmodified, reduced and carboxymethylated, or dansylated *E. coli* enzyme were studied by the method of Mann & Fish (1972). Lyophilized protein samples were dissolved in degassed 6.0 M guanidinium chloride, pH 8.6, and 0.1 M mercaptoethanol and chromatographed on Agarose A-5M (180 × 8 mm). Blue dextran and dansylalanine were included in each sample to measure the total exclusion and total inclusion volumes, respectively. Approximately 0.3-mL fractions were collected, and the exact volumes eluted were measured by weighing the fraction tubes in batches of five before and after elution. Proteins were monitored by absorption at 280 or 230 nm. Dansylated proteins were monitored at 240 nm.

Alkaline β-Elimination Test for O-Glycosidic Linkage. Alkaline β-elimination was carried out according to Bhavanandan et al. (1964) to test for O-glycosidic linkage to serine or threonine. Samples of purified enzyme were kept at 4 °C for 72 h in 0.5 N NaOH while control samples were kept in pH 7.0 Tris buffer. Both test samples and controls were then dialyzed against deionized water and lyophilized to dryness. Amino acid analysis was carried out as described, and the samples were compared for serine and threonine content.

Preparation of Polyspecific Antibody. Polyspecific antisera against fraction III enzyme were prepared in 3-month-old female rabbits. Proteins was suspended in Freunds' complete adjuvant. Subcutaneous injections were made at each of four sites (50–100 μg/site) every 10 days for a total of four sets of injections. The sites were located midway between each shoulder or hip joint and the spine. Rabbits were bled 5 days after the last set of injections. Blood was allowed to stand in glass tubes until clotting occurred (1–2 h at room temperature) and then was transferred to a cold room at 4 °C for 24 h to allow contraction of the clot. The straw-colored serum was removed and stored at 4 °C with 0.1% sodium azide. The γ-globulin fraction was prepared from this serum by ammonium sulfate fractionation (Campbell et al., 1970).

Preparation of Monospecific Antibody. Preparative double diffusion was carried out as described by Crowle (1973). When polyspecific antibody (prepared as described above) was diffused against fraction III enzyme, multiple precipitin bands were produced. The largest of these bands was identified as PRE–antibody complex by simultaneously diffusing the polyspecific antibody against apparently homogeneous fraction V enzyme. This produced a single, heavy band which was continuous with the main band produced between the polyspecific antibody and fraction III enzyme. The PRE–antibody complex identified in this way was removed, pooled, and then mixed with adjuvant and injected into fresh rabbits as described in the preparation of polyspecific antisera above. Three weeks after the first set of injections the rabbits were injected with fraction V enzyme. This method results in a large secondary response to the antigen given in the first set of injections but only a weak primary response (if any) to any antigenic trace contaminants in the preparation used for the second set of injections (Crowle, 1975).

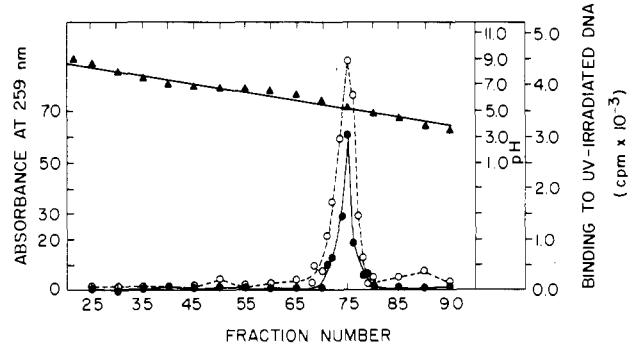

FIGURE 1: Profiles of absorbance at 257 nm (○) and binding to UV-irradiated DNA (●) of *E. coli* photoreactivating enzyme after isoelectric focusing. The pH profile of the column (▲) is also shown.

Absorption Spectra. Absorption spectra were recorded with a Cary 118C spectrophotometer with a scattered transmission accessory (Varian Associates, Model 1862000). Spectra were recorded either on paper and corrected for scattering by the method of Jagger (1967) or by a Cary 118C under the control of a Tektronix 4051 graphic system (Sutherland & Boles, 1977). These spectra were processed by the Tektronix, including correction for light scattering by the method of Latimer & Eubanks (1962), and plotted on a Versatec plotter (Xerox Corp.).

Results

Purification of the Enzyme. To carry out physical and chemical studies on a labile enzyme, it is first necessary to have a gentle and reliable method for purifying large quantities of that enzyme. We have developed such a method for the *E. coli* photoreactivating enzyme. The method is based on the streptomycin and ammonium sulfate precipitation steps of a purification procedure (method II) developed by Sutherland et al. (1973) but avoids the acetone and ion-exchange procedures which occasionally gave large losses of activity. Enzyme purified through ammonium sulfate precipitation (fraction III) routinely had specific activities of 500–600 units/mg.

This fraction was further purified by isoelectric focusing; the fractions collected from the isoelectric focusing column were monitored for pH, absorbance, and binding to UV-irradiated DNA. A typical profile is shown in Figure 1. The large peak of the absorbance and binding was coincident with photoreactivating enzyme activity. The fractions in the peak were pooled and chromatographed on Bio-Gel P-30 in buffer E. The elution profile for a representative P-30 column is shown in Figure 2; the major absorbance peak coincides with the peak of specific binding to UV-irradiated DNA. The resulting enzyme was designated fraction V. This fraction contained photoreactivating activity at a specific activity of about 2500 units/mg. Other preparations have had specific activities from 2000 to 4000 units/mg. This variability may result from the tendency of the enzyme to aggregate and/or loss of cofactor (see below).

Criteria of Homogeneity. We have used three major tests of the homogeneity of the enzyme preparation. First, fraction V enzyme shows only a single precipitin band when tested by double diffusion against a polyspecific antibody preparation which recognized multiple components in fraction III (ammonium sulfate precipitate) enzyme (see Figure 3). Second, sedimentation of fraction V enzyme at dilute concentrations in nondenaturing conditions gave a single symmetric peak. In addition, electrophoresis of dansyl-labeled enzyme gave only

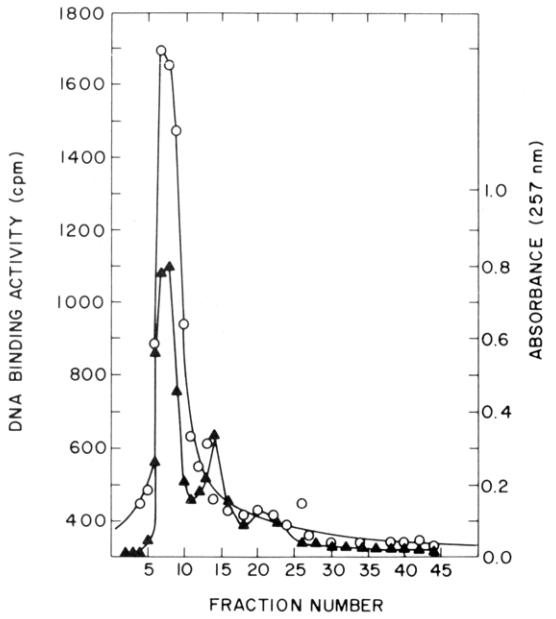

FIGURE 2: Chromatography of pooled photoreactivating enzyme from isoelectric focusing on a 2.5 × 50 cm Bio-Gel P-30, 50–100 mesh, column. Profiles are shown for absorbance at 257 nm (O) and binding to UV-irradiated DNA (▲).

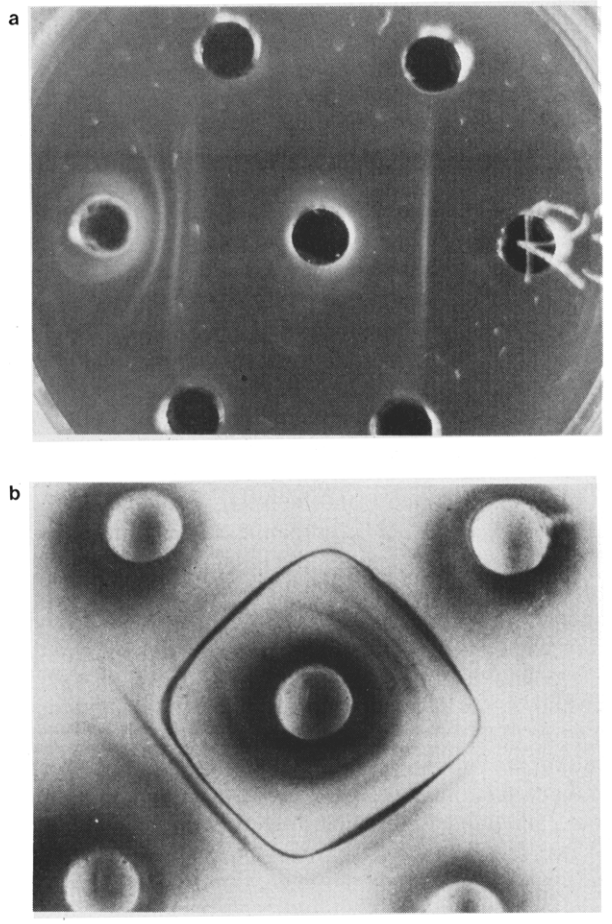

FIGURE 3: (a) Double-diffusion test of polyspecific antiserum (center well) prepared in response to fraction III enzyme against apparently homogeneous fraction V enzyme (right well) and fraction III enzyme (left well). (b) Double-diffusion test of monospecific serum (upper left well) and three polyspecific sera by reaction with fraction III partially purified enzyme.

bands corresponding to the molecular weight expected for the monomer of the enzyme (35 200 ± 200) plus its aggregates (see below).

Table I: Amino Acid Composition of *E. coli* Photoreactivating Enzyme

amino acid	mol of residues per mol of enzyme (to the nearest integer)
Lys	16
His	6
Arg	17
Asp + Asn	34
Ser	18
Thr	15
Glu + Gln	36
Pro	11
Gly	28
Ala	29
Val	23
Met	6
Ile	15
Leu	27
Tyr	8
Phe	9
Trp	0

Three other lines of evidence are consistent with the apparent homogeneity of fraction V enzyme. First, the absence of tryptophan from the amino acid analysis (see below) indicates the absence of contaminating proteins which would be expected to contain tryptophan. In addition, the amino acid analysis for fraction V enzyme was the same as that obtained for apparently homogeneous enzyme purified by the hydroxylapatite chromatographic method of Sutherland et al. (1973). Second, only one amino-terminal residue was detected by the method of Woods & Wang (1967). Finally, the specific activity of enzyme prepared by isoelectric focusing is in the same range as that prepared by DNA–cellulose chromatography, hydroxylapatite chromatography, or chromatography on triethylaminoethylcellulose (Sutherland et al., 1973).

Constituents of the Enzyme. The *E. coli* enzyme purified by the present method or by any of the three methods of Sutherland et al. (1973) contains both a protein and a nonprotein component.

(*1*) *The Apoprotein.* Because of the unusual nature of the reaction catalyzed by the enzyme, we first examined the apoprotein to determine its amino acid composition and the presence of any unusual or modified amino acids which might participate in the photolysis reaction. Table I shows the amino acid composition of fraction V enzyme. The enzyme is generally low in aromatic amino acids. Although the analysis was carried out in the presence of phenol and thioglycolic acid to protect aromatic residues, no tryptophan was detected. We thus examined the enzyme for the fluorescence (Longworth, 1971) and magnetic circular dichroic (Holmquist & Vallee, 1978) spectra characteristic of tryptophan. Tests of the Jobin-Yvon fluorometer indicated that even with 2-nm excitation slits, at a excitation wavelength of 288 nm with 352-nm emission, tryptophan could be easily detected at 10^{-6} M. However, examination of one enzyme preparation (3.5×10^{-5} M), before and after digestion with α-chymotrypsin and Pronase (Sasaki et al., 1975), indicated fluorescence only at a level which, if entirely due to tryptophan emission, would correspond to only 0.1–0.2 Trp/enzyme molecule. We also examined the magnetic circular dichroic spectra of the same enzyme preparation; although calibration tests indicated that the spectrometer could detect 1×10^{-6} M Trp in the presence of RNA of the same absorbance as was present in the enzyme sample, only small signals corresponding to about 0.2 Trp/enzyme molecule were detected; these measurements indicate that the preparation examined probably contained traces of

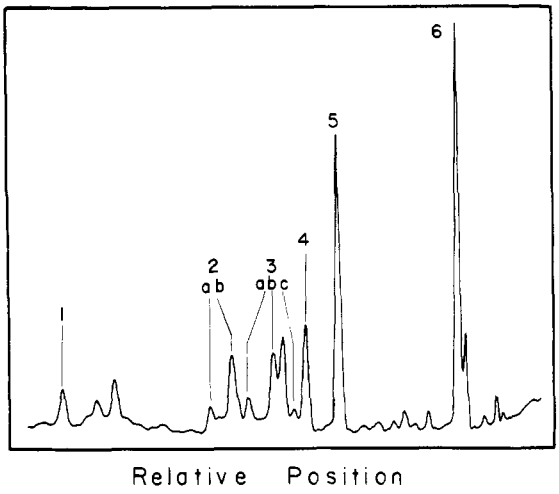

FIGURE 4: Gas–liquid chromatography of carbohydrate derived from *E. coli* photoreactivating enzyme. Peaks were identified by comparison with standards as follows: 1, *N*-acetylglucosamine; 2a and 2b, glucose; 3a–c, galactose; 4, mannose; 5, arabinose internal standard; 6, ribose.

tryptophan, presumably in proteins.

We also determined the NH_2-terminal amino acid by the method of Woods & Wang (1967). Only one amino-terminal residue (arginine) was detected in preparations of enzyme purified by either isoelectric focusing or hydroxylapatite chromatography.

(*2*) *Carbohydrate Associated with the Apoprotein.* The tendency of the enzyme to self-associate, seen in its precipitation during isoelectric focusing, reluctance to enter polyacrylamide gels, and concentration-dependent association during sedimentation equilibrium ultracentrifugation, led us to examine the enzyme for the presence of carbohydrate. Glycoproteins commonly show such aggregation, and Gottschalk (1972) pointed out that many glycoproteins form a "viscoelastic matrix" when concentrated in free electrophoresis or in electrophoresis in supporting media; the matrix is a type of gel itself and will not enter a separating gel when polyacrylamide electrophoresis is attempted. We divided fraction IV precipitated enzyme into two fractions, a "most soluble fraction" which resolubilized easily upon suspension in 10 mM Tris, pH 7.0, and "less soluble fraction" which remained precipitated after such treatment.

These fractions were first tested for carbohydrate content by the anthrone reaction (Mokrash, 1954). The most soluble fraction of the enzyme was found to be about 2% carbohydrate (grams of glucose equivalent/grams of protein). The values for this soluble fraction varied from 0.1 to 2.5% in different preparations. As a control, hen ovalbumin was measured as 1.3–1.4% carbohydrate (cf. 3.5% neutral sugar; Marshall & Neuberger, 1972). The less soluble fraction of *E. coli* photoreactivating enzyme was found to be 7.4–7.9% carbohydrate by the anthrone reaction.

The absorption spectra of the anthrone reaction products depend on the reacting carbohydrates (Herbert et al., 1971). We thus compared the spectra of the high and low carbohydrate fractions (i.e., the less soluble and most soluble fractions, respectively) and found that both had broad absorption bands with maxima at about 620 nm. This suggested that the monosaccharide composition was probably similar in the two fractions, the only difference being in the total amount present.

We next examined the hydrolyzed and trimethylsilylated carbohydrate derived from these fractions by gas–liquid chromatography (GLC). The analysis revealed the presence of mannose, glucose, galactose, and *N*-acetylglucosamine (Figure 4). Ribose was also seen, but only if the sample had been prepared for GLC analysis by dialysis in dialysis tubing with a molecular weight cutoff of 6000–8000 (Spectrapore 1). If a heat precipitation step was used for this protein and dialysis was carried out in large pore tubing (Spectrapore 2, molecular weight cutoff 12000–14000), there was a complete loss of ribose but no decrease in the other sugars. The highest value obtained for ribose was 14 mol/mol of enzyme (also see below).

Table II: Carbohydrate Associated with *E. coli* Photoreactivating Enzyme (High Carbohydrate Fraction)

monosaccharide	μg/mg of enzyme	mol/mol of enzyme
mannose	36.2	5
galactose	47.8	7
glucose	21.4	3
N-acetylglucosamine	23.2	3

The carbohydrate content of the less soluble fraction of *E. coli* photoreactivating enzyme was found to be up to 13% by GLC analysis. The most soluble fraction was about 0.6% carbohydrate. The same monosaccharides were seen in both the high and low carbohydrate fractions of the enzyme and in the same ratios. However, the low carbohydrate fraction did not have enough carbohydrate to allow accurate quantitation of the individual monosaccharides. Table II shows the monosaccharide composition of the high carbohydrate fraction.

The presence of *N*-acetylglucosamine implies a covalent linkage of the carbohydrate to the amide nitrogen of an asparagine residue. In all glycoproteins studied to date in which the carbohydrate groups were attached through an N-glycosidic bond to the amide nitrogen of asparagine, the glycosyl partner has been found to be *N*-acetylglucosamine (Neuberger et al., 1972). The other type of carbohydrate–peptide linkage commonly found in glycoproteins is the O-glycosidic bond to serine or threonine. In all glycoproteins in which this type of linkage has been found, the glycosyl partner has been *N*-acetylgalactosamine or xylose (Hood et al., 1978). We found *N*-acetylglucosamine but not *N*-acetylgalactosamine by GLC analyses of *E. coli* photoreactivating enzyme.

Although O-glycosidic linkages involving *N*-acetylglucosamine and serine or threonine have not been found in glycoproteins studied to date, we carried out an alkaline β-elimination test for O-glycosidic linkage to serine or threonine (Bhavanandan et al., 1964). Such linkages are destroyed by treatment with mild base, with conversion of serine and threonine to α-aminoacrylic acid and α-aminocrotonic acid, respectively. A selective loss of serine and/or threonine as seen in amino acid analysis following exposure of the proteins to mild base is taken as evidence of O-glycosidic linkage. No loss of serine or threonine was seen in the base-treated sample as compared to an identical sample kept as a control at pH 7.0, thus ruling out an O-glycosidic linkage to serine or threonine. There is no simple test for N-glycosidic linkage to asparagine, but the presence of *N*-acetylglucosamine taken together with a negative β-elimination test provides evidence for such a linkage.

RNA Associated with the Enzyme. The enzyme (whether from *E. coli* B or the K-12 derivative W3350 λC1857S7 dg D ΔJ, purified by isoelectric focusing or ion-exchange chromatography) is also associated with material with an absorption maximum near 260 nm (Sutherland & Sutherland, 1974; Wun et al., 1977). This material was identified as RNA on the basis of lability to base hydrolysis, digestion by pancreatic RNase, and base composition; after acid hydrolysis, UV-absorbing materials with chromatographic mobilities in

several solvents identical with adenine, cytosine, guanine, and uracil (but no thymine) were observed. By use of an extinction coefficient (ϵ = 0.1%) of 25 mL/(mg cm) for RNA (Brownlee, 1972), it was estimated that there were 10 molecules of RNA base per molecule of PRE (fraction V, 35 200 daltons). In one such enzyme preparation, quantitative ninhydrin assays for proteins were used and gave a value of eight molecules of RNA base per enzyme molecule. Similar values have been obtained by using fluorescamine assays for protein determination (G. Cimino, personal communication).

Molecular Weight. The lability of the purified enzyme and its strong tendency to aggregate have been serious obstacles in the determinations of molecular weight. To avoid these problems, it was necessary to use either very dilute solutions or strongly denaturing conditions. Using these conditions, we analyzed the molecular weight of the enzyme by three methods.

(*1*) *Gel Electrophoresis.* $NaDodSO_4$–polyacrylamide gel electrophoresis with dansyl-prelabeled *E. coli* photoreactivating enzyme and molecular weight standards (Inouye, 1971) gave an intense fluorescent band at a position corresponding to a molecular weight of 35 200 ± 200 followed by progressively fainter bands at positions corresponding to molecular weights of 68 000 ± 2000, 107 000, and 164 000 ± 3500. The fourth band was not always seen. Since homogeneous dansylated bovine serum albumin runs as multimers in this system (Inouye, 1971), it seems likely that the higher molecular weight species correspond to molecular weight multiples of the enzyme monomer.

(*2*) *Gel Filtration.* Since $NaDodSO_4$ binding can be anomalous in the case of glycoproteins (Segrest & Jackson, 1972) and since *E. coli* photoreactivating enzyme preparations were known to contain carbohydrate, it was important to obtain a second estimate for the molecular weight under denaturing conditions not dependent on $NaDodSO_4$ binding. Molecular sieving on a calibrated Agarose A-5M column in 6 M guanidinium chloride gave a molecular weight of 35 200 for reduced and carboxymethylated *E. coli* PRE. Because of its low 280-nm absorption, it was necessary to follow the elution of PRE at 230 nm as recommended by Mann & Fish (1972) for proteins of low aromatic amino acid content. Dansylated *E. coli* enzyme was also chromatographed in the same system and its elution monitored by absorption at 240 nm (the peak of dansyl absorption in 6 M guanidinium chloride). The dansylated PRE eluted at a position corresponding to a molecular weight of 35 500.

(*3*) *Sedimentation Velocity Ultracentrifugation.* The fact that *E. coli* photoreactivating enzyme copurifies with RNA was exploited to allow analytical ultracentrifugation studies at very low protein concentrations. Samples used for analytical ultracentrifugation studies were taken from the leading edge of the peak of photoreactivating enzyme activity which eluted in the void volume of the molecular sieving column (Figure 2).

Sedimentation of the enzyme at 44 000 rpm in an AnD rotor of the Model E ultracentrifuge was followed by absorption at 263 nm. All of the absorption sedimented as a single symmetrical boundary, and sedimentation was linear with time throughout each experiment. (If samples were allowed to stand in centrifuge buffer, on ice, for more than a week, all activity was lost and less than half of this 263-nm absorbing material would sediment.) Sedimentation coefficients were corrected to water at 20 °C ($s_{20,w}$). Values of $s_{20,w}$ from experiments at six concentrations were extrapolated to zero concentration to give an $s^0_{20,w}$ of 3.72 S (correlation coefficient = −0.90921). The lowest protein concentration used for this extrapolation was 0.11 mg/mL and the highest was 0.38 mg/mL. Taken with the experimental molecular weight of 35 200, this value for $s_{20,w}$ suggests that the enzyme is approximately spherical ($f/f_0 \simeq 1$) in solution. If it is assumed that the $f/f_0 = 1$, a molecular weight of 36 800 can be calculated. The 8–10 mol of RNA base per mol of enzyme would, of course, account for a few percent of the mass.

Discussion

In spite of great interest in their specificities and modes of action, DNA repair enzymes remain largely uncharacterized. The small number of cellular copies of most repair enzymes, the interference of other activities with their assay, and their lability have made their study difficult. We have taken advantage of the unique specificity and light dependence of the photoreactivating enzyme and a high yield *E. coli* strain and have developed a gentle purification procedure to obtain large quantities of apparently homogeneous enzyme. We have used such preparations for a basic characterization of the *E. coli* photoreactivating enzyme.

These preparations are homogeneous by several criteria. First, only a single precipitin band is formed when fraction V enzyme is tested by double diffusion against a polyspecific antibody preparation which recognizes many components in partially purified (fraction III) enzyme (Figure 3a). (Note that Figure 3b shows the identity of the band recognized by polyspecific sera as PRE, the same species recognized by the monospecific serum.) Second, in dilute solutions the native enzyme sediments as a single symmetric peak during velocity sedimentation. Third, $NaDodSO_4$ gel electrophoresis of the dansylated enzyme shows only bands corresponding to the enzyme monomer and its higher multiples. In addition, the detection of only one amino-terminal residue is consistent with the absence of major contaminants in the preparation.

Sedimentation velocity measurements on the intact holoenzyme yield a monomer molecular weight of 36 800. Electrophoresis of dansyl-labeled photoreactivating enzyme in polyacrylamide gels under denaturing conditions showed a major fluorescent band corresponding to a molecular weight of 35 200 ± 200, plus bands appearing at 68 000, 107 000, and 164 000. The migration of dansylated bovine serum albumin as multimers in this system supports the notion that the higher molecular weight species are aggregates of the enzyme monomer. Since glycoproteins can show anomalous $NaDodSO_4$ binding, we also examined the reduced and carboxymethylated enzyme as well as the dansyl-labeled species by gel filtration in guanidinium chloride. The carboxymethylated derivative gave an apparent molecular weight of 35 200 and the dansyl derivative of 35 500.

The enzyme is associated with carbohydrate in varying amounts; gas–liquid chromatography indicated the presence of mannose, glucose, galactose, and *N*-acetylglucosamine in the apoprotein. The high carbohydrate content probably contributes to the aggregation of the enzyme, seen especially at high enzyme concentrations. The enzyme is also associated with RNA of approximately 10 nucleotides/enzyme molecule. Removal of the RNA by Norit adsorption results in stoichiometric loss of enzyme activity. Although the role of the RNA, if any, in enzyme action is not yet clear, Stark et al. (1978) have shown that ribonuclease P contains a RNA cofactor essential for enzyme activity. In addition, Rupert (1960) showed that the *E. coli* enzyme was composed of a nondialyzable and a dialyzable component. The ability of the *E. coli* enzyme, as well as that from baker's yeast, to be stimulated by several "activators" (Minato & Werbin, 1972) precludes

the use of regain of activity for absolute identification of a required cofactor.

Hélène has shown that at short UV wavelengths ($\lambda < 320$ nm), tryptophan in Lys-Trp-Lys or the Gene 32 protein of bacteriophage T4 can mediate photolysis of pyrimidine dimers in DNA (Hélène et al., 1976). Mortelmans et al. (1977) have suggested that "the observations of Hélène and co-workers raise the possibility that a variety of proteins (possibly characterized by high binding affinity for DNA) and even peptides can be confused with true photoreactivating enzymes both in vivo and in vitro".

However, amino acid analysis indicated that the enzyme is low in aromatics and did not show the presence of any tryptophan moieties. Although both phenol and thioglycolic acid were included during hydrolysis to protect tryptophan residues, it was possible that we might have failed to detect a few or a single residue. Since tryptophan gives large and characteristic signals in fluorescence and magnetic circular dichroism, we examined the spectral properties of the enzyme. Only trace signals were detected, at much less than 1 Trp/PRE molecule. We conclude that the *E. coli* photoreactivating enzyme probably lacks tryptophan, thus making it unlikely that photoreactivation of dimers by this enzyme proceeds by tryptophan-mediated photolysis.

Acknowledgments

We thank Dr. R. C. Warner for guidance in the sedimentation experiments, C. Painter and A. Shih for preparation of enzyme, C. Fuselier for collaboration in the early stages of this work, W. Hanes for collaboration in preparation of antibodies, and Dr. J. C. Sutherland for critical reading of the manuscript.

References

Barth, G., Records, R., Bunnenberg, E., Djerassi, C., & Voelter, W. (1971) *J. Am. Chem. Soc. 93*, 2545–2547.

Bhavanandan, V. P., Buddecke, E., Carubelli, R., & Gottschalk, A. (1964) *Biochem. Biophys. Res. Commun. 16*, 353–357.

Bohlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Brownlee, G. G. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., & Work, E., Eds.) Vol. 3, p 22, North-Holland Publishing Co., New York.

Campbell, D. H., Garvey, J. S., Cremer, N. E., & Sussdorf, P. H. (1970) *Methods in Immunology*, 2nd ed., W. A. Benjamin, New York.

Cleaver, J. E. (1968) *Nature (London) 218*, 652–656.

Crowle, A. J. (1973) *Immunodiffusion*, 2nd ed., Academic Press, New York.

Crowle, A. J. (1975) *Methods of Protein Separation* (Catsimpoulas, N., Ed.) Vol. VI, pp 69–92, Plenum Press, New York.

Gottschalk, A., Ed. (1972) *Glycoproteins*, part A, pp 42 and 45, Elsevier Publishing Co., New York.

Harm, W. (1964) *Mutat. Res. 8*, 411–415.

Hart, R., Setlow, R. B., & Woodhead, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5574–5578.

Hélène, C., Toulme, F., Charlier, M., & Yanin, M. (1976) *Biochem. Biophys. Res. Commun. 71*, 91–98.

Herbert, D., Phipps, P. J., & Stramp, R. E. (1971) in *Methods in Microbiology* (Norris, J., Ed.) Vol. 5B, pp 266–336.

Holmquist, B., & Vallee, B. L. (1978) *Methods Enzymol. 49*, Part G, 149–179.

Hood, L. E., Weisman, I. L., & Wood, W. B. (1978) *Immunology*, Benjamin-Cummings Publishing Corp., Menlo Park, CA.

Inouye, M. (1971) *J. Biol. Chem. 246*, 4834–4838.

Jagger, J. (1967) *Introduction to Research in Ultraviolet Photobiology*, Prentice-Hall, Englewood Cliffs, NJ.

Kondo, S., & Kato, T. (1966) *Photochem. Photobiol. 5*, 827–837.

Latimer, P., & Eubanks, C. A. (1962) *Arch. Biochem. Biophys. 98*, 274–285.

Layne, E. (1957) *Methods Enzymol. 3*, 447–454.

Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–484, Plenum Press, New York.

Madden, J. J., Werbin, H., & Denson, J. (1973) *Photochem. Photobiol. 18*, 441–445.

Mann, K. G., & Fish, W. W. (1972) *Methods Enzymol. 27*, 28–42.

Marshall, R. D., & Neuberger, A. (1972) in *Glycoproteins* (Gottschalk, A., Ed.) pp 732–761, Elsevier Publishing Co., New York.

Minato, S., & Werbin, H. (1972) *Physiol. Chem. Phys. 4*, 467–476.

Mokrash, L. C. (1954) *J. Biol. Chem. 208*, 55–59.

Mortelmans, K., Cleaver, J. E., Friedberg, E. C., Paterson, M. C., Smith, B. P., & Thomas, G. H. (1977) *Mutat. Res. 44*, 433–446.

Neuberger, A., Gottschalk, A., Marshall, R. D., & Spiro, R. G. (1972) in *Glycoproteins* (Gottschalk, A., Ed.) pp 450–490, Elsevier Publishing Co., New York.

Rupert, C. S. (1960) *J. Gen. Physiol. 42*, 573–595.

Rupert, C. S. (1962) *J. Gen. Physiol. 45*, 725–741.

Sasaki, T., Abrams, B., & Horecker, B. L. (1975) *Anal. Biochem. 65*, 396–404.

Segrest, J. P., & Jackson, R. L. (1972) *Methods Enzymol. 28*, Part B, 54–63.

Setlow, J. K., Boling, M. E., & Bollum, F. J. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1430–1436.

Setlow, R. B. (1963) *Science (Washington, D.C.) 153*, 379–386.

Setlow, R. B. (1973) *Novas Tendencias em Fotobiologia, Suplemento dos Anais da Academia Brasileira de Ciencias 45*, 215–220.

Setlow, R. B., Regan, J. C., German, J., & Carrier, W. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1035–1041.

Shapiro, A. L., Vinuela, E., & Maizel, J. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.

Spackman, D. H., Moore, S., & Stein, W. H. (1958) *Anal. Chem. 30*, 1190–1206.

Stark, B. C., Kole, R., Bowman, E. J., & Altman, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3717–3721.

Sutherland, B. M., & Chamberlin, M. J. (1973) *Anal. Biochem. 53*, 168–176.

Sutherland, B. M., Carrier, W. L., & Setlow, R. B. (1970) *Science (Washington, D.C.) 158*, 1699–1700.

Sutherland, B. M., Court, D., & Chamberlin, M. J. (1972) *Virology 48*, 87–93.

Sutherland, B. M., Chamberlin, M. J., & Sutherland, J. C. (1973) *J. Biol. Chem. 248*, 4200–4205.

Sutherland, J. C., & Sutherland, B. M. (1974) in *Progress in Photobiology* (Schenk, G. O., Ed.) pp 21–25, Deutsche Gesellschaft fur Lichtfaschung, Frankfurt.

Sutherland, J. C., & Boles, T. (1977) *Rev. Sci. Instrum. 48*, 19–23.

Sutherland, J. C., Cimino, G. D., & Lowe, J. T. (1976) *Rev. Sci. Instrum. 47*, 358–360.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871–872.
Weber, K., & Osborn, M. (1975) in *The Proteins* (Neurath, H., Hill, R. L., & Boeder, C.-L., Eds.) 3rd ed., Vol. I, pp 179–223, Academic Press, New York.
Woods, K. R., & Wang, K. T. (1967) *Biochim. Biophys. Acta 133*, 369–370.
Wun, K. L., Gih, A., & Sutherland, J. C. (1977) *Biochemistry 16*, 921–924.

Elementary Steps in the Reaction Mechanism of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*: Kinetics of Acetylation and Deacetylation†

Steven K. Akiyama‡ and Gordon G. Hammes*

ABSTRACT: The kinetics of the acetylation of the pyruvate dehydrogenase complex from *Escherichia coli* by [3-^{14}C]pyruvate and of the deacetylation of the complex by coenzyme A have been studied by using rapid mixing–quench techniques. The time course for acetylation in 4 mM thiamin pyrophosphate, 2 mM $MgSO_4$, and 0.02 M potassium phosphate (pH 7.0) at 4 °C can be analyzed in terms of two kinetic processes. At long times 10 nmol of acetyl groups is incorporated per mg of enzyme complex (48 sites per complex of molecular weight 4.8×10^6). The slower process is much too slow to be of catalytic significance. The rate constant for the faster process is not dependent on enzyme concentration and reaches a limiting value of 40–65 s^{-1} at high pyruvate concentrations; the exact value is dependent on the detailed acetylation mechanism assumed. The minimum molar turnover number of the enzyme complex is 420 s^{-1} (17.5 s^{-1} per pyruvate decarboxylase). The acetylated lipoic acids are deacetylated by coenzyme A at a rate much faster than that of acetylation. Complete deacetylation is obtained only if the deacetylation is carried out within seconds of the acetylation, apparently because dead-end intramolecular transfers of acetyl groups from the lipoic acids to other functional groups on the enzyme not essential for catalytic activity can occur. The results obtained suggest only about half of the acetylation reactions are on the main catalytic pathway.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* consists of three different enzymes (Koike et al., 1963; Willms et al., 1967) with a polypeptide-chain stoichiometry of 24 pyruvate decarboxylases (E_1),[1] 24 dihydrolipoyl transacetylases (E_2), and 12 dihydrolipoyl dehydrogenases (E_3) and an overall molecular weight of 4.8×10^6 (Reed, 1974; Angelides et al., 1979). These enzymes catalyze the decarboxylation of pyruvic acid and the acetylation of coenzyme A by the following sequence of reactions (Koike et al., 1960):

$$\text{pyruvate} + E_1[\text{TPP}] \rightarrow CO_2 + E_1[\text{hydroxyethyl-TPP}] \quad (1)$$

$$E_1[\text{hydroxyethyl-TPP}] + E_2[\text{Lip-(S)}_2] \rightleftharpoons E_1[\text{TPP}] + E_2[\text{HS-Lip-S-acetyl}] \quad (2)$$

$$E_2[\text{HS-Lip-S-acetyl}] + \text{coenzyme A} \rightleftharpoons E_2[\text{Lip-(SH)}_2] + \text{acetyl coenzyme A} \quad (3)$$

$$E_2[\text{Lip-(SH)}_2] + E_3[\text{FAD}] \rightleftharpoons E_2[\text{Lip-(S)}_2] + E_3[\text{FAD(red.)}] \quad (4)$$

$$E_3[\text{FAD(red.)}] + NAD^+ \rightleftharpoons E_3[\text{FAD}] + \text{NADH} + H^+ \quad (5)$$

where TPP, Lip-$(S)_2$, and Lip-$(SH)_2$ are thiamin pyrophosphate and oxidized and reduced lipoic acid, respectively. The brackets indicate cofactors and intermediates which are tightly or covalently bound to the enzyme.

The mechanism of the overall reaction involves the interaction of a lipoic acid with the catalytic sites of all three enzymes. Resonance energy transfer measurements suggest that a single lipoic acid cannot span the distances between the catalytic sites (Moe et al., 1974; Shepherd & Hammes, 1976, 1977) and that lipoic acid residues are in close proximity to each other (Angelides & Hammes, 1979). Studies of the overall activity of chemically modified enzyme complexes indicate that at least two lipoic acids are required per catalytic cycle and that only about seven active lipoamide dehydrogenases are required for a completely active complex (Angelides & Hammes, 1978). Thus, the mechanism for the overall reaction very likely involves the transfer of acetyl groups and oxidation–reduction between lipoic acids (Brown & Perham, 1976; Bates et al., 1977; Collins & Reed, 1977; Angelides & Hammes, 1978).

For further elucidation of the reaction mechanism, a study of the individual steps in the reaction mechanism utilizing fast reaction techniques has been initiated. In this work the acetylation and deacetylation of the complex (eq 1–3) have been studied by using quenched stopped-flow techniques. The acetylation of the lipoic acids occurs by two pathways, with only one of the pathways being sufficiently rapid to be involved in the overall catalysis. The rate of acetylation via the faster pathway reaches a limiting value at high pyruvate concentrations, 0.02 M potassium phosphate (pH 7.0), and 4 °C, which is greater than the minimum turnover number of the complex under identical conditions. In the catalytic cycle, the rate of deacetylation of the lipoic acids by coenzyme A is much faster than the rate of acetylation; furthermore, pathways exist

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received November 30, 1979.* This work was supported by grants from the National Institutes of Health (GM 13292) and the National Science Foundation (PCM77-11392).

‡ National Institutes of Health Predoctoral Trainee (GM 00834 and GM 07273).

[1] Abbreviations used: E_1, pyruvate decarboxylase; E_2, dihydrolipoyl transacetylase; E_3, dihydrolipoyl dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide.

0006-2960/80/0419-4208$01.00/0