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Escherichia coli Dihydrofolate Reductase: Isolation and Characterization of Two Isozymes†

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ABSTRACT: A combination of affinity column chromatography and preparative gel electrophoresis has been used to purify to homogeneity the two isozymes of dihydrofolate reductase from a trimethoprim-resistant strain of *Escherichia coli* B (RT 500). These enzyme forms are noninterconvertible and are present in crude cell lysates, but other electrophoretic species can be generated during purification if sulfhydryl-protecting agents, such as dithiothreitol, are not present. The two isozymes, numbered form 1 and form 2 with respect to their decreasing electrophoretic mobilities, have similar molecular weights (18 500), molecular radii (21 Å), and apparent K_m values for reduced nicotinamide adenine dinucleotide (NADH) and NADH phosphate (NADPH). Both forms contain 2 mol of sulfhydryl/mol of enzyme which can be oxidized to intramolecular disulfide bonds. However, forms 1 and 2 differ physically in their electrophoretic mobility and isoelectric point and kinetically in their pH–activity profile, specific activity, K_m for dihydrofolate, and their affinity toward a number of inhibitors.

Multiple forms of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3.) have been isolated from a variety of bacterial and mammalian sources. In some cases the nature of the multiplicity is understood. For example, the two forms of dihydrofolate reductase from *Lactobacillus casei* (Gundersen et al., 1972), chicken liver (Huennekens et al., 1971), and L1210 cells (Perkins et al., 1967) that were observed on electrophoresis and column chromatography were identified as the free enzyme and the enzyme-NADPH[1] binary complex. The multiple forms of dihydrofolate reductase isolated from several strains of *Streptococcus faecium* var. durans A have been shown to be genetically determined isozymes with different physical and kinetic properties (Nixon and Blakley, 1968; Albrecht et al., 1969), and it has been recently shown (Amyes and Smith, 1975; Sköld and Widh, 1975) that R factors can introduce into *Escherichia coli* a trimethoprim-resistant enzyme that is significantly different from the chromosomal enzyme. However, in other cases, such as with the dihydrofolate reductase from hamster kidney cells (Hänggi and Littlefield, 1974), *Diplococcus pneumoniae* (Sirotnak, 1973), and bovine liver (Kaufman and Kamerer, 1976; Baumann and Wilson, 1975), the occurrence of polymorphism is well documented, while the cause of the multiplicity is not completely understood.

We previously reported the *E. coli* enzyme can exist as aggregates and monomers with different electrophoretic mobilities (Baccanari et al., 1975a). The purpose of the present study was to determine the basis for this polymorphism and to characterize the multiple enzyme forms. This report shows two of the multiple forms are isozymes with different physical and kinetic properties. These isozymes can exist in a variety of sulfhydryl oxidation states which results in a complex electrophoretic pattern. A mechanism for the sulfhydryl interconversions and the relationship of these findings to other studies of dihydrofolate reductase are also presented.

Materials and Methods

Iodoacetic acid, Nbs_2, $PhCH_2SO_2F$, and soybean trypsin inhibitor were from Sigma. Acrylamide and bisacrylamide were purchased from Eastman Kodak. Folate and DTT were from Calbiochem, and NADPH was from P-L Biochemicals. Ampholine-carrier ampholytes were supplied by LKB. The protein standards used in the Ferguson analysis were from several commercial sources. The α-lactalbumin (mol wt

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; $NADP^+$, NAD phosphate; NADPH, reduced NAD phosphate.

14 400; radius 19.3 Å), and DNase I (mol wt 31 000; radius 23.7 Å) were from Sigma. The α-chymotrypsinogen B (mol wt 25 000; radius 22.2 Å) was a contaminant of α-chymotrypsinogen A obtained from Worthington. Myoglobin (mol wt 17 000; radius 20.8 Å) and ovalbumin (mol wt 43 500; radius 27.2 Å) were from Schwarz/Mann. Methotrexate was from Nutritional Biochemicals, and all other inhibitors were synthesized at the Wellcome Research Laboratories. Dihydrofolate was prepared by the method of Futterman (1957) as modified by Blakley (1960) and stored as a suspension in 5 mM HCl at −70 °C.

Enzyme Assays. The dihydrofolate reductase assays and the determinations of the I_{50} values were performed in 0.1 M imidazolium chloride buffer, pH 7 (Baccanari et al., 1975a). In determining the apparent K_m for dihydrofolate, the NADPH concentration was 60 μM. The mercaptoethanol concentration varied with dihydrofolate, but 24 mM was the highest mercaptoethanol concentration used. The apparent K_m for NADPH was determined in 0.1 M imidazolium chloride, pH 7, containing 90 μM dihydrofolate and 24 mM mercaptoethanol. The reported kinetic values are the average of at least two separate determinations. All kinetic experiments were performed with homogeneous forms 1 or 2 obtained by preparative electrophoresis. Upon isolation, forms 1 and 2 had specific activities of 85 units/mg and 7.4 units/mg, respectively, under the standard assay conditions.

Enzyme Preparation. Dihydrofolate reductase was purified from RT 500 *E. coli* B using several modifications of the procedure previously described (Baccanari et al., 1975a). By using this modified scheme, smaller quantities of the protein were purified in 7 days rather than 30 days, thereby decreasing the probability of proteolysis or other modifications which can occur in crude extracts. Also, the proteolytic enzyme inhibitor $PhCH_2SO_2F$ (10 μM) was included in the initial lysis buffer. *E. coli* RT 500 (20 L), grown in the absence of trimethoprim, was harvested and lysed, and the crude extract was subjected to ammonium sulfate fractionation in 1 day. The protein fraction that was soluble at 45% saturated ammonium sulfate and precipitated at 90% saturated ammonium sulfate (4250 units of enzyme, 2000 mg of protein) was dissolved in 100 mL of 50 mM potassium phosphate buffer, 0.2 M KCl, 0.5 mM EDTA, 1 mM DTT, pH 6, and then mixed with 6 mL of Methotrexate-Sepharose resin, placed in a dialysis bag, and dialyzed overnight against the same buffer. In this manner, 80–90% of the dihydrofolate reductase activity was bound to the resin in 12 h. The resin was batch washed two times with 0.2 M potassium phosphate buffer, 1 M KCl, 1 mM DTT, pH 6, placed in a column, and then washed and eluted at a flow rate of 5 mL/h (Baccanari et al., 1975a). By using this method, about 2500 units of enzyme (45 mg of protein) was recovered. The protein content of the purified enzyme determined by fluorescent Methotrexate titration (Perkins and Bertino, 1966) agreed within 98% of the Lowry (Lowry et al., 1951) protein value.

Polyacrylamide Gel Electrophoresis. Analytical gel electrophoresis was performed according to the method of Ornstein (1964). About 10 μg of protein was applied to each gel. Gels for Ferguson analysis were cast using appropriate dilutions of a stock acrylamide solution to ensure constant % C while varying % T. Some gels were stained to detect carbohydrate by using the periodic acid–Schiff technique of Zacharius et al. (1969).

Preparative gel electrophoresis was carried out in a Canalco Prep-Disc apparatus using the same discontinuous system as in the analytical gels. The column surface area was 1.8 cm^2 with a 10-cm separating gel and a stacking gel volume equal to the volume of the sample applied. The sample was layered under the cathode buffer, and electrophoresis was conducted using an ISCO Model 492 power supply in the constant power mode. The initial 4-W power corresponds to approximately 330 V at 12 mA. After the sample had stacked and reached the stacking gel–separating gel interface, the power was increased to 10 W (675 V and 14.8 mA). The flow rate of the elution buffer (0.37 M Tris-Cl, pH 8.9, 20% (v/v) glycerol, and 1 mM DTT) was maintained at 1.5 mL/min and two fractions/min were collected.

Sodium dodecyl sulfate gel electrophoresis was performed using the method of Laemmli (1970). The protein bands were visualized with Coomassie brilliant blue according to the method of Fairbanks et al. (1971).

Isoelectric Focusing. Isoelectric focusing was performed in 6-cm polyacrylamide gels (5% T, 2.5% C) as described by Righetti and Drysdale (1971). The gels were prefocused at constant current (1 mA/gel) for 30 min. Samples, in 20 mM potassium phosphate buffer (pH 6.9) containing 10% ampholine (pH 3.5–10), were loaded, focused at 1 mA/gel for 6 h, and stained for protein (Malik and Berrie, 1972). The pH gradient was measured by slicing unstained control gels into 5-mm sections which were macerated and eluted overnight in 0.5 mL of 0.01 M KCl.

Sulfhydryl Analysis. The sulfhydryl group contents of the various enzyme samples were determined using the method of Habeeb (1972). Pool A (26 units/mg) and pool B (81 units/mg) from the affinity chromatography step were dialyzed exhaustively against degassed 20 mM potassium phosphate, pH 7, under a N_2 atmosphere. The final reaction mixture contained approximately 40 μg of enzyme, 0.5 mg/mL EDTA, 1% sodium dodecyl sulfate in 1.0 mL of 0.1 M sodium phosphate buffer, pH 8. Nbs_2 (50 μL of a 6.7 mM solution in 0.2 M sodium phosphate, pH 8) was added to the above mixture and the moles of free sulfhydryl was calculated from the net change in absorbance at 412 nm and the molar extinction coefficient 13 600 M^{-1} cm^{-1}. In some cases the sodium dodecyl sulfate was omitted from the reaction. The molar concentration of enzyme used in each determination was calculated from its protein concentration and an enzyme molecular weight of 18 500. Results presented are the average of three determinations.

Results

Enzyme Purification. The rapid purification of dihydrofolate reductase resulted in an increased recovery compared to the slower procedure (70 vs. 52%, Baccanari et al., 1975a). More importantly, $NaDodSO_4$ gel electrophoresis of the purified enzyme resulted in only one band of protein with a molecular weight of 18 500. The minor protein band (10%) with a molecular weight of 16 500 previously observed on $NaDodSO_4$ electrophoresis (Baccanari et al., 1975a) was absent. Analytical gel electrophoresis of the entire pool from the affinity column gave results similar to gel B in Figure 1. About 80% of the protein was found in a rapidly migrating species called form 1. The remaining 20% was in a band that migrated more slowly and is called form 2. No other protein-staining bands were present.

Figure 1 shows the final step in the enzyme purification, elution of the affinity column with folic acid. The enzymatic activity did not elute as a symmetrical peak. Rather, there was a shoulder on the leading edge. Therefore, the elution profile was divided into three major fractions: pool A (leading edge), pool B (peak), pool C (trailing edge). Electrophoresis of pool A showed it contained 20% form 1 and 80% form 2 (gel A, Figure 1). Pool B contained 80% form 1 and 20% form 2 (gel

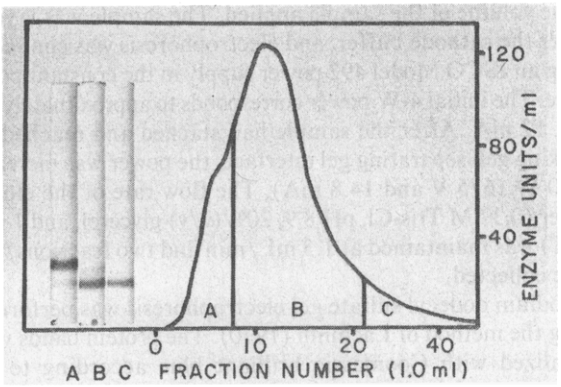

FIGURE 1: Partial separation of forms 1 and 2 by affinity chromatography. A 6-mL methotrexate affinity column containing 3600 units of dihydrofolate reductase was prepared and washed as described in Methods. At fraction 0, the column was eluted with 0.2 M potassium phosphate buffer, 1 M KCl, 1 mM DTT, and 3 mM folate, pH 8, at a flow rate of 5 mL/h. Each fraction was assayed for enzyme activity and those corresponding to A, B, and C were pooled and subjected to electrophoresis. The gels, stained for protein, are shown on the left with their origin (—) on the top.

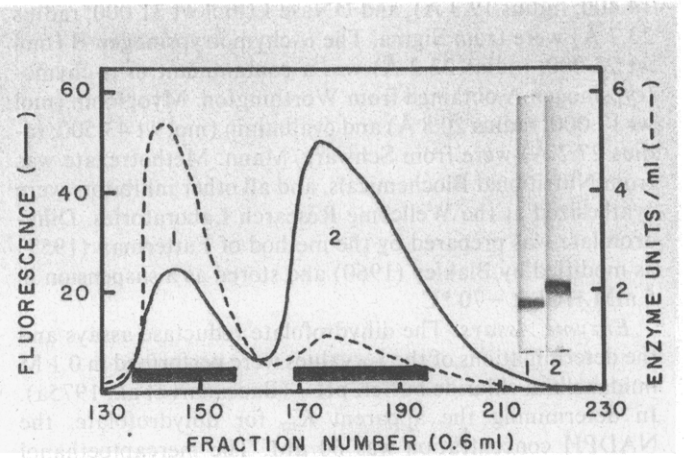

FIGURE 2: Separation of forms 1 and 2 by preparative electrophoresis. Pool A from the affinity column (108 units of enzyme and 4 mg of protein in 2.7 mL) was incubated with 0.3 mL of 100 mM DTT for 1 h at 5 °C, and then 0.5 mL of 40% sucrose and 40 μL of 0.01% bromophenol blue were added immediately before electrophoresis. Collection of fractions was started shortly before the bromophenol blue marker migrated off the column. The fractions were monitored both for enzymatic activity and intrinsic protein fluorescence (excitation, 290 nm; emission, 340 nm), and those indicated by the horizontal bars were pooled. About 60% of the applied enzymatic activity and protein was recovered. Enzyme from pools 1 and 2 was subjected to analytical electrophoresis and stained for protein (gels on right).

B). Pool C contained only form 1 (gel C). Therefore, forms 1 and 2 appear to differ in their affinity for Methotrexate, and one can be enriched with respect to the other by proper pooling of the affinity column elution fractions.

Pools A, B, and C were dialyzed against 50 mM potassium phosphate buffer, 1 mM DTT, pH 7, filter sterilized (Nucleopore, 0.2-μm pore diameter), and stored at 5 °C. Greater than 90% of the activity remained after 4 weeks. The electrophoretic mobilities of the purified enzymes were not altered by ammonium sulfate precipitation or lyophilization. However, protein bands with electrophoretic mobilities different from forms 1 and 2 appeared after storage without DTT. This observation will be discussed in detail below.

Preparative Gel Electrophoresis. Attempts to separate form 1 from form 2 by gradient-elution affinity chromatography were unsuccessful. However, separation could be achieved by preparative gel electrophoresis (Figure 2). The protein elution profile corresponds to the protein-stain intensity observed on analytical electrophoresis of the same sample (i.e., gel A, Figure 1). However, it is evident from Figure 2 that the ratio of enzymatic activity to protein (fluorescence) of form 1 is greater than that of form 2, suggesting the isozymes differ in specific activity. This was verified when the specific activities of forms 1 (85 units/mg) and 2 (7.4 units/mg) at pH 7 were calculated from protein values determined by Methotrexate titrations (Perkins and Bertino, 1966). Analytical electrophoresis of the pooled fractions (Figure 2, gels 1 and 2) shows that forms 1 and 2 were completely separated by this procedure. Both isozymes were stable in 50 mM potassium phosphate buffer, pH 7, 20% glycerol, 1 mM DTT. Greater than 75% of the original activity remained after 6 weeks at 5 °C.

Forms 1 and 2 Are Not Interconvertible. When multiple forms of an enzyme are observed, the question arises whether or not one form is generated from the other during purification. This question could be easily answered for *E. coli* RT 500 dihydrofolate reductase, because the organism produces sufficient quantities of the enzyme such that forms 1 and 2 could be detected early in the purification scheme at the ammonium sulfate precipitation step. Both forms 1 and 2 were observed on analytical or preparative gel electrophoresis of these unpurified preparations. Furthermore, electrophoresis of the crude enzyme before and after the affinity gel binding step showed both forms were bound to the Methotrexate resin. It is assumed that the unequal distribution between the forms after elution of the affinity column is due to loss of the more loosely bound form 2 during the column washing that precedes the folate elution step.

Other factors which could potentially cause epigenetic enzyme multiplicity were also examined. Both forms 1 and 2 were detected in approximately equal amounts in (1) crude extracts of cloned cells harvested in early log, late log, or stationary phase; (2) cells disrupted by Brij lysis, sonication, or French press; (3) cells grown in glucose minimal medium or a rich medium (Trypticase Soy Broth, Difco); (4) cells grown in the presence or absence of 350 μg/mL trimethoprim. Also, no interconversion between purified forms 1 and 2 was observed upon 6-weeks storage at pH 7, 24 h at pH 2 or 10, lyophilization, ammonium sulfate precipitation or incubation with folate, dihydrofolate, NADPH, NADH, $NADP^+$, NAD^+, Methotrexate or trimethoprim. Forms 1 and 2 have similar UV absorption spectra, with an absorption maximum at 282 nm and a shoulder at 290 nm. The low absorbance between 320 and 360 nm indicates the preparations are free of ligands that absorb in this region (NADPH, dihydrofolate or folate). The fluorescence emission spectra of forms 1 and 2 are also similar, showing a single emission peak at 338 nm when excited at 282 nm. Neither form contains detectable carbohydrate when assayed by the periodic acid–Schiff technique.

Molecular Weight, Molecular Size, and Isoelectric Point. The separation of proteins on gel electrophoresis is dependent upon molecular size (molecular weight) and charge. A series of experiments was designed to determine the contribution of each parameter in the separation of forms 1 and 2. Sephadex G-100 gel filtration (1.5 × 90 cm) of a mixture of the isozymes in 0.1 M potassium phosphate, 1 M KCl, pH 8, indicated both species have the same molecular weight (about 20 000). Also, an analysis of forms 1 and 2 electrophoretic mobility was performed using a technique first described by Ferguson (1964) and later refined by Rodbard and Chrambach (1971). In this procedure proteins with the same size but different charge will give parallel lines when the logarithm of electro-

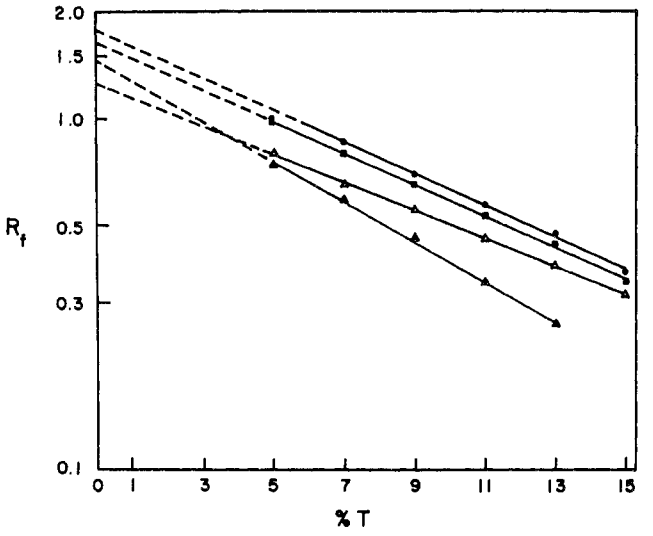

FIGURE 3: Ferguson analysis of forms 1 and 2. Form 1 (●) and form 2 (■) dihydrofolate reductase, DNase (▲), and α-lactalbumin (Δ) were subjected to electrophoresis in gels varying in acrylamide concentration from 5 to 15% T. The relative electrophoretic mobilities were determined, and the unweighted regression lines were computed from the logarithms of the mobilities. The calculated slopes (K_r values) are: form 1 dihydrofolate reductase, −0.045; form 2 dihydrofolate reductase, −0.045; DNase, −0.057; and α-lactalbumin, −0.040.

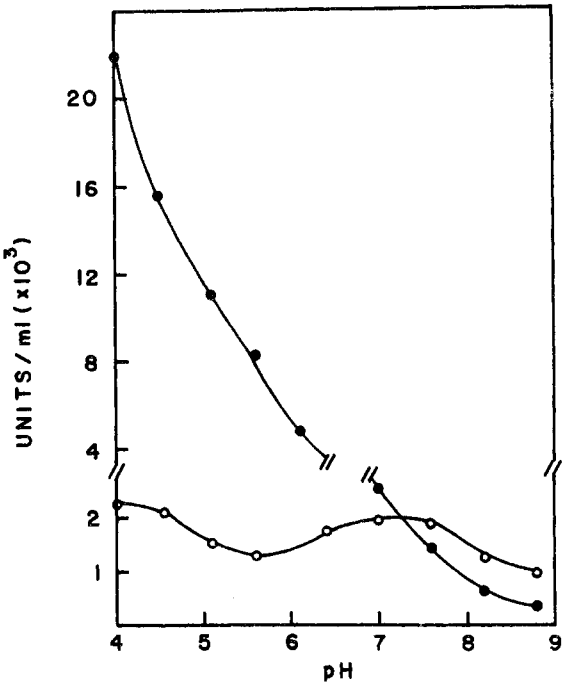

FIGURE 4: pH–activity profiles. Form 1 (○) and form 2 (●) dihydrofolate reductase were assayed at 30 °C in 0.1 M Tris-Cl (pH 7.6–8.8), 0.1 M imidazolium chloride (pH 6.1–7.6), and 0.1 M succinate-Tris (pH 4.0–6.1). Each assay contained 40 μM dihydrofolate, 12 mM 2-mercaptoethanol, 60 μM NADPH, and either 37 ng of form 1 or 347 ng of form 2 in a final volume of 1.0 mL. The amounts of forms 1 or 2 used were those necessary to give nearly equal enzyme activity (about 2.2×10^{-3} units/mL) at pH 7. Corrections were made for nonenzymatic changes in absorbance at acidic pH values.

phoretic mobility (R_f) is plotted vs. acrylamide concentration (% T). Proteins with different sizes will result in nonparallel lines. Data plotted in this manner are presented in Figure 3. The two forms of dihydrofolate reductase have lines with identical slopes (−0.045). The Ferguson plot of DNase is included to illustrate the slope (K_R value) of a molecule with almost twice the molecular weight of dihydrofolate reductase (31 000 vs. 18 500), and α-lactalbumin (mol wt 14 400) is included to illustrate the sensitivity of the method.

The electrophoretic system used in the Ferguson analysis was calibrated (Gonenne and Lebowitz, 1975) by plotting log mol wt or log Stokes' radius vs. slope (K_R) for a series of well characterized proteins (listed in Materials and Methods). By using this method, it was determined that forms 1 and 2 have identical molecular radii and molecular weights of 21 Å and 19 700, respectively.

Sodium dodecyl sulfate gel electrophoresis showed both forms comigrated as a single band with a calculated molecular weight of 18 500. Therefore, all data indicate the two enzyme forms have identical molecular weights and molecular radii and differ only by charge. This charge difference was verified by gel electrofocusing. Form 1, which has the faster electrophoretic mobility at pH 8.9, has an isoelectric point of pH 4.6, whereas form 2 has an isoelectric point of pH 4.7.

Kinetic Properties. The pH–activity profiles of forms 1 and 2, determined in Tris-Cl, imidazolium chloride, and succinate-Tris buffers, are strikingly different (Figure 4). Form 1 appears to have a double pH optimum with similar enzymatic activities at pH 4 and 7. Form 2 has no detectable pH "optimum". Its enzymatic activity continually increases as the pH is decreased from pH 9 to 4. Therefore, although form 1 has about a tenfold greater specific activity than form 2 at pH 7 (55 vs. 6.9 units/mg), they have nearly equal specific activities at pH 4 (61.5 and 64.2 units/mg, respectively).

Isoenzymes often differ in their substrate and coenzyme specificities. For example, two dihydrofolate reductases are isolated from a Methotrexate-resistant mutant of *S. faecium* var. durans; one utilizes dihydrofolate as a substrate, whereas the other can utilize either folate or dihydrofolate (Nixon and Blakley, 1968; Albrecht et al., 1969). However, *E. coli* RT 500 forms 1 and 2 are both dihydrofolate reductases; neither utilize folate efficiently. V_{max} values with folate as a substrate are 800- to 1000-fold less than those with dihydrofolate at pH 4.5. Also, both forms 1 and 2 have relatively poor affinities for NADH with apparent K_m values of 0.4 to 0.6 mM. The apparent K_m values for the preferred substrates, dihydrofolate and NADPH, are shown in Table I. Although both isozymes have similar K_m values for NADPH (2.3–4.4 μM), form 2 has a significantly lower K_m for dihydrofolate (0.65 μM) than form 1 (8.9 μM). Table I also compares the affinities of a number of dihydrofolate reductase inhibitors for forms 1 and 2. All are assumed to be competitive inhibitors,[2] and the K_i values were calculated from I_{50}, the dihydrofolate concentration in the assay (40 μM), and the dihydrofolate K_m (Cheng and Prusoff, 1973). In each case, form 2 is less sensitive to inhibition than form 1, and the differences in binding ranged from 80-fold for trimethoprim to fivefold for pyrimethamine.

Sulfhydryl Oxidation and the Generation of Other Electrophoretic Forms. The occurrence of enzymes with various sulfhydryl oxidation states is a common cause of multiplicity (Baccanari et al., 1975b; Blackburn et al., 1972). In fact, sulfhydryl groups have already been implicated in the generation of an aggregated dihydrofolate reductase (Baccanari et al., 1975a). Therefore, it was of interest to determine the free sulfhydryl group content of forms 1 and 2. The following experiments were performed with affinity column pool A (80% form 2, 20% form 1) and pool B (80% form 1, 20% form 2) rather than the electrophoretically separated species. It will

[2] Trimethoprim was shown by double-reciprocal plots to be a competitive inhibitor of dihydrofolate for forms 1 and 2 with kinetically determined apparent K_i values of 1.2 and 127 nM, respectively.

TABLE I: Kinetic Properties of Forms 1 and 2 Dihydrofolate Reductase.

	Apparent K_m (μM)[a]		Apparent K_i (nM)[b]			
	FH_2	NADPH	A	B	C	D
Form 1	8.9 ± 2.1	4.4 ● 1.0	1.3	200	27	15
Form 2	0.65 ± 0.1	2.3 ± 0.5	106	1060	260	270

[a] The values for apparent K_m are the average of two determinations. In each determination the standard error was less than 17%. [b] Abbreviations used are: A, 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (trimethoprim); B, 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (pyrimethamine); C, 2,4-diamino-6-butylpyrido(2,3-d)pyrimidine hydrochloride; D, 2,4-diamino-6-ethylquinazoline hemihydrate.

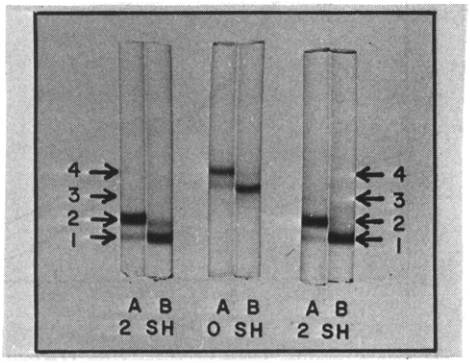

FIGURE 5: Generation of reducible forms 3 and 4. Dihydrofolate reductase (50 μg/mL), from affinity column pools A and B, were exhaustively dialyzed against degassed 20 mM potassium phosphate buffer, pH 7. Lowry (1951) proteins and Nbs_2 titrations were performed on each sample to determine the sulfhydryl-group content, and aliquots were subjected to gel electrophoresis and then stained for protein (left two gels). The enzymes were heated at 65 °C for 35 min, and samples were again taken for sulfhydryl determinations and electrophoresis (center two gels). The heat-treated preparations were then incubated with 10 mM DTT (final concentration) for 1 h at room temperature and then exhaustively dialyzed against degassed 20 mM potassium phosphate buffer, pH 7, under nitrogen. The dialyzed enzyme was subjected to electrophoresis and protein and Nbs_2 titrations were again performed (right two gels).

become evident that the conclusion was not biased by this choice. Nbs_2 titrations of forms 1 and 2 showed them to contain 1.9 mol of sulfhydryl/mol of enzyme and 2.0 mol of sulfhydryl/mol of enzyme, respectively. In each case the same value was obtained whether or not $NaDodSO_4$ was present in the reaction mixture. Those samples lacking $NaDodSO_4$ required about 35 min for the reaction to be completed, as opposed to the almost instantaneous reaction when $NaDodSO_4$ was present.

When the enzyme was purified without DTT, two additional protein bands appeared upon electrophoresis of stored preparations. In most cases, preincubation of these preparations with DTT before electrophoresis resulted in the disappearance of the added bands with an accompanying increase in the amounts of forms 1 and 2. The addition of DTT to freshly isolated enzyme prevented the appearance of the altered enzyme forms. Therefore, it seemed forms 1 and 2 could be converted to other electrophoretic species by sulfhydryl oxidation. It was subsequently found that heating the enzyme resulted in complete conversion of forms 1 and 2 to their separate oxidized species called reducible form 3 and reducible form 4, respectively (Figure 5, left and center). Nbs_2 titration of heat-treated forms 1 and 2 showed reducible forms 3 and 4 contained 0.22 mol of sulfhydryl/mol of enzyme and no detectable sulfhydryl, respectively. These forms are called reducible because they can be converted back to species with the same mobilities as forms 1 and 2 by incubation with DTT (Figure 5, right). Ferguson analysis of reducible forms 3 and 4 showed they had the same Stokes' radii (and molecular weight) as forms 1 and 2 and therefore are not aggregation products formed via intermolecular disulfide bonds. Reducible forms 3 and 4 have no measurable enzymatic activity, but it is not known if this is due to a change in enzyme conformation upon oxidation or thermal inactivation during heating.

Discussion

The evidence presented in this study shows *E. coli* RT 500 contains two dihydrofolate reductase isozymes, forms 1 and 2, that have different physical and kinetic properties. Both species are present in crude lysates of cloned cells. This approximately equal distribution of enzyme protein remains constant when the physiological state of the cell is altered by changing the composition of the culture medium or the phase of growth when the cells are harvested. Even when the isozymes are purified to homogeneity, neither form is converted to the other by changes in ionic strength, ammonium sulfate precipitation, lyophilization, or high or low pH. These are conditions which would be expected to accelerate nonenzymatic modifications of a protein. Therefore, it is unlikely that one species is an epigenetically modified form of the other generated either while the cells are growing or during the enzyme purification. The physical properties of forms 1 and 2, determined by gel filtration, Ferguson analysis, $NaDodSO_4$ electrophoresis, and isoelectric focusing, have shown them to be charge isozymes. They have the same molecular weight and Stokes' radius, but differ 0.1 pH unit in isoelectric point. Preliminary evidence on the structural differences between forms 1 and 2 shows the isozymes have sufficiently different amino acid sequences to yield at least one tryptic fragment that migrates differently upon both chromatography and electrophoresis (data not shown). Kinetically, the isozymes have different K_m values for dihydrofolate, K_i values for a variety of inhibitors, and greatly different pH–activity profiles.

RT 500 dihydrofolate reductase was originally prepared by a more time consuming purification scheme (Baccanari et al., 1975a) in which the enzyme was extensively washed while bound to the affinity column. These preparations had the same pH–activity profiles and kinetic properties as form 1. Our inability to detect form 2 in these earlier preparations could be due to the weaker binding of this isozyme to the Methotrexate resin and its loss during the affinity chromatography step. Because of this, the enzyme used for sequence determination (Stone et al., 1976) and inhibitor-binding studies (Pattishall et al., 1976) was predominately form 1. Polymorphism is also apparent with the dihydrofolate reductase from another strain of *E. coli*. Although the enzyme isolated from *E. coli* MB 1428 was originally shown to exist as a single electrophoretic form (Poe et al., 1972) a reexamination using the Ornstein (1964) electrophoresis system showed the enzyme preparation contains multiple protein forms with two of the bands having mobilities closely corresponding to RT 500 forms 1 and 2 (D. Baccanari, unpublished). Further characterizations of these multiple forms have not been made.

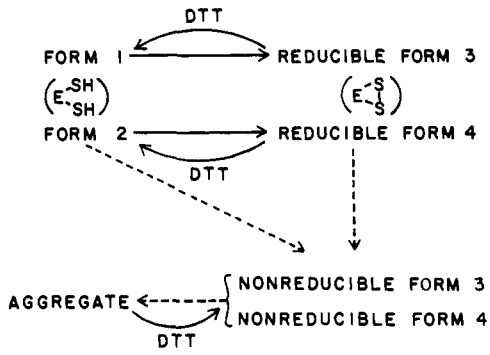

FIGURE 6: Scheme representing the interrelationships between the various forms of dihydrofolate reductase. Proven interconversions are indicated by solid arrows. Likely, but unproven, interconversions are indicated by the broken arrows.

Forms 1 and 2 both contain 2 mol of sulfhydryl/mol of enzyme. Oxidation of these sulfhydryls to intramolecular disulfide bonds leads to the generation of two species with lower electrophoretic mobilities than forms 1 and 2. These enzymes are called reducible forms 3 and 4, and they can be converted back to forms 1 and 2 by DTT. We have also observed upon heating or prolonged storage in the absence of DTT the generation of small amounts of protein with electrophoretic mobilities similar to reducible forms 3 and 4 but which cannot be converted to forms 1 or 2 by DTT (data now shown). We call these proteins nonreducible forms 3 and 4. Ferguson analysis shows they are monomers (D. Averett, unpublished), but little more is known except they were also present in preparations purified by the longer purification scheme (Baccanari et al., 1975a). An aggregated form of the enzyme also exists (Baccanari et al., 1975a). The aggregate has only been observed in preparations containing nonreducible forms 3 and 4, and it can be completely disrupted to nonreducible forms 3 and 4 by DTT (data not shown). These complex interconversions are summarized in Figure 6. Since we are uncertain if nonreducible forms 3 and 4 are generated from reducible forms 3 and 4 or from forms 1 and 2, both pathways are represented. Also, the mechanism by which DTT converts the aggregate to nonreducible forms 3 and 4 has not been established.

Enzyme sulfhydryl-group oxidation states are important in other dihydrofolate reductases. For example, studies in our laboratory (S. Smith, personal communication) show that mercaptoethanol or DTT protects the porcine liver enzyme from inactivation during storage. Baumann and Wilson (1975) recently obtained direct evidence for the reactivity of sulfhydryls in the bovine liver enzyme. They observed a high-molecular-weight form of dihydrofolate reductase (about 20% of the total enzyme) on $NaDodSO_4$ gels and column chromatography. This species was disrupted by mercaptoethanol, and they suggested two monomers could be linked by an intermolecular disulfide bond. Also, the monomer was shown to contain an intramolecular disulfide bond that could be reduced by DTT. However, they did not compare the electrophoretic mobilities of these oxidized and reduced enzymes. Like the enzyme from *E. coli* RT 500, the dihydrofolate reductase from *E. coli* MB 1428 contains 2 mol of sulfhydryl/mol of of enzyme (Williams and Hoogsteen, 1974). Electrophoresis of a preparation from this source that was purified in the absence of sulfhydryl-protecting agents shows multiple forms with mobilities comparable to the *E. coli* RT 500 reducible and nonreducible forms 3 and 4 (D. Baccanari, unpublished). However, purification of the enzyme in the presence of thiol-protecting agents eliminates this type of multiplicity (M. Poe, personal communication). Therefore, the proposed mechanism for the interconversions we observed with the *E. coli* RT 500 enzyme (i.e., Figure 6) should be useful in studying other dihydrofolate reductases.

As dihydrofolate reductases are being purified from an increasing number of organisms, the evidence mounts that multiplicity is not a unique phenomenon. However, the identification of the enzymes from *S. faecium* var. durans A as separate folate and dihydrofolate reductases (Nixon and Blakley, 1968; Albrecht et al., 1969) is the only case where a physiological role for the isozymes is known. Our attempts to find different substrate specificities or changes in the amounts of forms 1 or 2 synthesized under different growth conditions were unsuccessful. Further work will involve identifying a physiological role for the isozymes and examining wild-type *E. coli* for their presence.

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Thermal Unfolding Transition of Ribonuclease A Measured by 2′-CMP Binding†

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ABSTRACT: We report an approach to the problem of detecting and characterizing intermediates in the unfolding of ribonuclease A. Two distinct properties of the protein are compared at equilibrium within the unfolding transition zone: (1) a physical property of the protein, the absorbance of buried tyrosine residues, and (2) a functional property, the ability to bind the specific ligand, 2′-CMP. A direct comparison of these two properties is made within the pH 5.8 transition zone, and an indirect comparison is made by using the stopped-flow instrument to sample rapidly the equilibrium properties of the pH 2.0 transition. At both pH 2.0 and pH 5.8, the results indicate that there are no intermediates in folding which have the physical properties of the native enzyme but which have lost the ability to bind a specific ligand.

When the heat-induced unfolding transition of RNase A[1] is measured by different physical properties (Harrington and Schellman, 1956; Ginsburg and Carroll, 1965; Brandts and Hunt, 1967; Tsong et al., 1970; Tiktopulo and Privalov, 1974; Chen and Lord, 1976), the transition is found to be highly cooperative. Transition curves for unfolding at pH 2.0 are found to be superimposable when measured by viscosity, optical rotation, or tyrosine absorbance (Ginsburg and Carroll, 1965), and the two-state approximation ($N \rightleftharpoons U$) has been used to describe the unfolding transition curves (Brandts and Hunt, 1967). Laser Raman measurements suggest that noncoincident unfolding curves may be observable by this technique, but greater accuracy is needed to be certain (Chen and Lord, 1976). NMR measurements of thermal unfolding curves of individual protons for the four histidine residues show a single unfolding curve at pH 4.0 (Matthews and Westmoreland, 1973). At the acidic pH 1.3, the unfolding curve of His-12 precedes by 1° the common curve of the other three residues (Westmoreland and Matthews, 1973), probably because His-12 is involved in a local unfolding reaction (see also Benz and Roberts, 1975).

There are two possible explanations as to why it is difficult to detect intermediates in unfolding at equilibrium above pH 1.3: (1) either intermediates are populated at levels too low to be detectable or (2) the intermediates have physical properties close to those of native RNase A or of its unfolded form. The second explanation may appear unlikely: however, it has been shown to be correct in the case of an intermediate detected in kinetic experiments. There are two forms of unfolded RNase A that have very different rates of refolding (Garel and Baldwin, 1973, 1975a,b; Brandts et al., 1975; Hagerman and Baldwin, 1976) and the fast-refolding species U_2 is an intermediate in the folding of the slow-refolding species U_1 (Hagerman and Baldwin, 1976). Most physical properties of U_1 and U_2 are indistinguishable, but a small pK difference has been detected (Garel and Baldwin, 1975b). Moreover, the equilibrium ratio $(U_2)/(U_1)$ does not depend significantly on temperature (Garel and Baldwin, 1973, 1975a), so that, even if a thermal unfolding curve could be measured by a property that is very different for U_2 and U_1, this curve would still coincide with the unfolding curves measured by tyrosine absorbance or viscosity.

Consequently, it is necessary to scrutinize carefully the experiments which have failed to detect any intermediates with properties close to N. Such intermediates would be expected to retain the compact structure of N but to show some local unfolding. A "quasi-native" intermediate has been reported recently in the guanidine-induced unfolding of α-lactalbumin (Kuwajima et al., 1976). In the case of RNase A, if a comparable quasi-native intermediate exists it should have been detected in kinetic studies of unfolding (cf. Hagerman and Bal-

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A, disulfide bonds intact; NMR, nuclear magnetic resonance; N, native; U, unfolded.