enzymes, dGuo/dAdo kinase and dCyd/dAdo kinase, and has a specific activity on either protein which is less than one-fourth that of the opposite domain. However, when the dGuo and dCyd sites are saturated, activity at the dAdo kinase site increases severalfold, so that the regulated combined output of the two bifunctional pairs may be envisioned as the approximately equal quantities of dAMP, dCMP, and dGMP needed for DNA synthesis. A separate thymidine kinase, also subject to both positive and negative controls (Durham & Ives, 1971), supplies the fourth deoxynucleotide, dTMP.

Registry No. dGuo, 961-07-9; dAdo, 958-09-8; FSO_2BzAdo, 57454-44-1; dGuo/dAdo kinase, 89618-28-0; dCyd/dAdo kinase, 75302-35-1.

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Chorismate Mutase/Prephenate Dehydrogenase from *Escherichia coli* K12: Purification, Characterization, and Identification of a Reactive Cysteine†

Graham S. Hudson,* Vic Wong,‡ and Barrie E. Davidson

ABSTRACT: The bifunctional enzyme involved in tyrosine biosynthesis, chorismate mutase/prephenate dehydrogenase, has been isolated from extracts of a regulatory mutant of *Escherichia coli* K12. The pure enzyme is a homodimer of total molecular weight 78 000 and displays Michaelis–Menten kinetics for both activities. Fingerprinting and amino acid sequencing of tryptic and thermolytic peptides of the S-[^{14}C]carboxymethylated enzyme allowed the identification of three unique cysteine-containing sequences per subunit. Chemical modification of the native enzyme with 5,5′-dithiobis(2-nitrobenzoate) or iodoacetamide showed that one sulfhydryl group per subunit was particularly reactive, and the integrity of this group was essential for both enzymic activities. This work supports previous proposals for a close spatial relationship between the active sites.

Chorismate mutase/prephenate dehydrogenase (EC 5.4.99.5/1.3.1.12) is a bifunctional enzyme (Cotton & Gibson, 1965; Koch et al., 1971a) catalyzing two consecutive steps in the biosynthesis of tyrosine in *Escherichia coli* K12 and other enteric bacteria (Figure 1). Chorismate, the product of the common aromatic pathway, is rearranged to form prephenate, which subsequently undergoes oxidative decarboxylation to yield (4-hydroxyphenyl)pyruvate, the keto acid equivalent of tyrosine. The activity of this enzyme is regulated by repression of its synthesis (Brown & Somerville, 1971; Im et al., 1971) and end-product inhibition (Koch et al., 1971a). The enzyme was first purified from *E. coli* and *Aerobacter aerogenes* by Koch et al., who showed it to be a bifunctional dimer of closely similar or identical subunits (Koch et al., 1970a,b, 1971a,b). Chorismate mutase/prephenate dehydratase, a similar enzyme involved in the biosynthesis of phenylalanine (Figure 1), has also been characterized as a bifunctional dimer of identical subunits (Davidson et al., 1972; Gething & Davidson, 1976).

Chemical modification studies have been used to examine both the nature of the groups involved in the functions of these enzymes and the relationship between the active sites of the two activities of each enzyme. For chorismate mutase/prephenate dehydratase, Gething and Davidson (1977a,b) established that the reaction of approximately 1 mol of sulfhydryl group/subunit with either 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2)[1] or *N*-ethylmaleimide caused the loss of all of the prephenate dehydratase activity but of only a small percentage of the chorismate mutase activity. It was concluded that a sulfhydryl group is essential for the prephenate dehydratase

† From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia. *Received May 2, 1984.* This work was supported by Grants D72/17662 and D2-77/15417 from the Australian Research Grants Scheme. G.S.H. was in receipt of a Commonwealth Postgraduate Research Award during this work.

* Address correspondence to this author at the CSIRO Division of Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia.

‡ Present address: Silenus Laboratories, Dandenong, Victoria, Australia.

[1] Abbreviations: Cmc, 5-(carboxymethyl)cysteine; dansyl chloride, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); SDS, sodium dodecyl sulfate.

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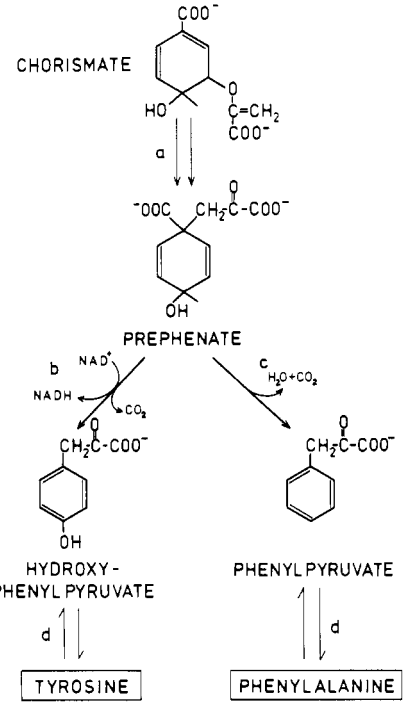

FIGURE 1: Tyrosine and phenylalanine pathways of *E. coli* K12. Enzymes: a and b = chorismate mutase/prephenate dehydrogenase; a and c = chorismate mutase/prephenate dehydratase; d = aromatic aminotransferase.

activity and that the enzyme has a separate active site for the chorismate mutase activity. Preliminary investigations on the sulfhydryl groups of chorismate mutase/prephenate dehydrogenase from *E. coli* and *A. aerogenes* have indicated that modification of these groups is associated with a parallel loss of both enzymic activities (Koch et al., 1972; Heyde, 1979). Thus, the two activities of this enzyme may be catalyzed at the same site or at closely situated active sites.

Our aims were to conduct structural investigations of chorismate mutase/prephenate dehydrogenase, in particular to examine the reactivity of the sulfhydryl groups and their requirement for enzymic activity (this paper) and the mechanism of inhibition by tyrosine (Hudson et al., 1983). The purification procedure of Koch et al. (1971a) was found to be slow and gave poor yields of an unstable product. We report (i) the development of a new and rapid purification scheme for the isolation of stable enzyme necessary for these studies, (ii) characterization of the enzyme and comparison with the properties of chorismate mutase/prephenate dehydratase, and (iii) the identification of the three unique cysteines per subunit, in particular, a reactive cysteine associated with the two activities. SampathKumar & Morrison (1982) have also recently reported a new purification scheme for this enzyme.

Experimental Procedures

Materials. Chorismate and prephenate were prepared as described previously (Gibson, 1968; Dudzinski & Morrison, 1976). Blue Dextran–Sepharose 4B was prepared by the method of Ryan & Vestling (1974). Thermolysin was a gift of Dr. T. A. A. Dopheide of the C.S.I.R.O. Division of Protein Chemistry, Parkville, Victoria, Australia. Iodo[2-^{14}C]acetate and iodo[1-^{14}C]acetamide were from the Radiochemical Centre, Amersham, U.K., and their specific activities were determined after isotopic dilution by reaction with excess cysteine at pH 8.5 and 37 °C, treatment with acid to convert *S*-[(carboxyamido)methyl]cysteine to *S*-(carboxymethyl)-cysteine (Cmc), purification of the product by electrophoresis at pH 2.1, amino acid analysis, and liquid scintillation counting. All other chemicals were obtained commercially.

Enzyme Assays. Chorismate mutase was assayed routinely in 0.4-mL reaction mixtures containing 1.0 mM chorismate in 0.1 mg/mL bovine serum albumin, 50 mM Tris-HCl, pH 7.5, 0.5 mM sodium EDTA, and 10 mM 2-mercaptoethanol, as described by Gething et al. (1976). Prephenate dehydrogenase was assayed in 0.4-mL reaction mixtures containing 0.2 mM prephenate and 2.0 mM NAD^+ in the same buffer. After 5-min reaction at 37 °C, the mixtures were diluted with 1.0 mM tyrosine, and their absorbances were read at 340 nm. Prephenate dehydratase was assayed by the method of Gething et al. (1976). Kinetic studies were performed as described by Hudson et al. (1983). A unit of enzyme is defined as the quantity of enzyme that catalyses the conversion of 1 μmol of substrate to product in 1 min under the assay conditions.

Bacterial Strains and Growth Conditions. E. coli strain JP2319, containing the *tyrR370* and *trpS378* alleles (Camakaris et al., 1980), was provided by Professor J. Pittard of the Microbiology Department of the University of Melbourne. The *tyrR370* allele leads to derepression of the *aroF/tyrA* operon (encoding, respectively, the tyrosine-sensitive isoenzyme of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase and chorismate mutase/prephenate dehydrogenase). The *trpS378* allele is a temperature-sensitive mutation of tryptophanyl-$tRNA_{Trp}$ synthetase. At 37 °C or above the instability of this enzyme leads to a decrease in the level of tryptophanyl-$tRNA_{Trp}$ and, in the presence of the *tyrR370* allele and limiting tryptophan, increased transcription of the *aroF/tyrA* operon. Growth of JP2319 on limiting tryptophan yields a level of chorismate mutase/prephenate dehydrogenase 50–100-fold above that of wild-type organisms and 3–5-fold above those of isogenic *trpS*$^+$ strains. JP2319 was grown at 37 °C in 12-L batches of minimal medium (Monod et al., 1951) supplemented with 0.5% glucose, 0.3 mM histidine, and 0.1% yeast extract (Difco), from a 200-mL inoculum previously grown at 30 °C. The cells were harvested at late log phase (10 h), yielding 3.5–4 g of cell paste/L of medium. The paste was stored at −20 °C for up to 1 year without loss of chorismate mutase/prephenate dehydrogenase activity.

Buffers. Buffers using *N*-ethylmorpholine and containing 1 mM sodium citrate were used in the purification of the enzyme following a report of improved stability of chorismate mutase/prephenate dehydrogenase in such buffers during purification (SampathKumar, 1978). Those used were as follows: buffer A, 50 mM Cl/*N*-ethylmorpholine, pH 7.5, 1 mM Na_2EDTA, 1 mM sodium citrate, and 10 mM 2-mercaptoethanol; buffer B, 25 mM Cl/*N*-ethylmorpholine, pH 7.1, 1 mM Na_2EDTA, 1 mM sodium citrate, and 10 mM 2-mercaptoethanol; buffer C, as buffer B but pH 8.0; storage buffer, buffer A with 0.02% sodium azide, 50% (vol/vol) glycerol, and 5 mM dithiothreitol in place of 2-mercaptoethanol.

Purification of Chorismate Mutase/Prephenate Dehydrogenase. All steps were done at 0–4 °C. (1) Frozen cells (200 g) were suspended in 1 L of buffer A plus 50 mM potassium chloride, broken at 100 kg cm^{-2} in a French pressure cell, and centrifuged at 16000*g* for 60 min. (2) Streptomycin sulfate (0.56 volume of a 10% aqueous solution) was added over 30 min to the supernatant with stirring, and after 1 h the suspension was centrifuged at 16000*g* for 45 min. (3) A 38–50% ammonium sulfate fraction was prepared by adding 0.21 g of solid ammonium sulfate/mL of supernatant, centrifuging, and adding a further 0.081 g/mL new supernatant. After recentrifugation the second pellet was dissolved in buffer

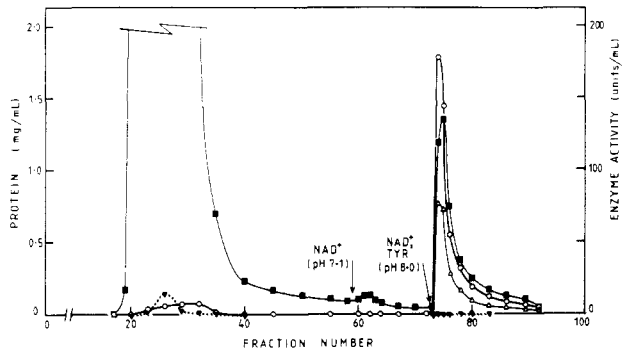

FIGURE 2: Chromatography of crude extracts of JP2319, containing chorismate mutase/prephenate dehydrogenase, on Blue Dextran/Sepharose. Conditions for chromatography are described under Experimental Procedures. Activities: (○) chorismate mutase; (△) prephenate dehydrogenase; (▼) prephenate dehydratase. (■) Protein.

A plus 10% glycerol, to give a final volume of 100 mL. (4) This was applied at 100–150 mL/h to a 5 × 48 cm column of Sephadex G-25 connected in series to a 2.6 × 24 cm column of Blue Dextran–Sepharose, both equilibrated with buffer B. After the elution of the protein from the Sephadex G-25 and prior to the elution of the salt fraction, the G-25 column was disconnected and the Blue Dextran–Sepharose was washed with 600 mL of buffer B followed by 300 mL of buffer B plus 1 mM NAD^+. The enzyme was eluted with buffer C containing 1 mM NAD^+ and 0.5 mM tyrosine (Figure 2). Simultaneous mixing of 1.0 M Tris-HCl, pH 7.1, 50% glycerol, and 10 mM 2-mercaptoethanol with the column effluent (1:5 by volume) helped stabilize the eluted enzyme from inactivation. After protein and enzyme assays, appropriate fractions were pooled, partially concentrated on an Amicon PM10 membrane to 50 mL, and dialyzed against buffer A plus 50% glycerol. (5) The enzyme was desalted on a 3.4 × 39 cm column of Sephadex G-25 equilibrated with buffer A plus 0.1 M potassium chloride at 100 mL/h. The flow rate was reduced to 20 mL/h, and the protein eluted directly onto a 1.5 × 88 cm column of DEAE-Sepharose CL-6B. After the DEAE-Sepharose was washed with 100 mL of the same buffer, the enzyme was eluted with a linear 600-mL chloride gradient (0.10–0.25 M potassium chloride in buffer A). The purified enzyme was concentrated to more than 2 mg/mL by ultrafiltration on a PM10 membrane and dialysis against storage buffer. It was stable in this buffer at −20 °C under nitrogen for 3 months.

Determination of Protein and Amino Acid Composition. The protein contents of purified enzyme samples were determined by amino acid analysis, while those of impure samples were obtained by the method of Bradford (1976) using bovine serum albumin as the standard. For amino acid analysis, samples were hydrolyzed and, after lyophilization, were run on a Beckman 121MB amino acid analyzer. Tryptophan was estimated by the method of Edelhoch (1967).

Molecular Weight Analysis. The native molecular weight of the enzyme was determined by sedimentation equilibrium analysis using the method of Yphantis (1964). Sedimentation velocity experiments were done at 59 780 rpm, using an Analytical D rotor and sector-shaped cells. The subunit molecular weight of the enzyme was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970).

S-[^{14}C]Carboxymethylation and Peptide Fingerprinting. Chorismate mutase/prephenate dehydrogenase (130 mg) was incubated in 25 mM dithiothreitol overnight at 4 °C and then desalted at 4 °C on Sephadex G-50 equilibrated with 10 mM Tris-HCl, pH 7.5, 5 mM Na_2EDTA, and 0.5 mM dithiothreitol. After pooling of the enzyme (to 60 μM subunits), one-tenth volume of 1.0 M Tris-HCl, pH 8.3, iodo[2-^{14}C]-acetate (equivalent to a 5-fold excess over protein sulfhydryl groups after reaction with the dithiothreitol), and solid guanidine hydrochloride (to 4 M) were added. The solution was incubated at 37 °C for 4 h under nitrogen and then dialyzed against 0.5% ammonium bicarbonate at 20 °C. Three proteolytic digestions were performed and then lyophilized: (a) 98 mg of S-[^{14}C]carboxymethylated enzyme was digested with 2 mg of trypsin at 37 °C for 4 h; (b) 10 mg was digested with 0.2 mg of thermolysin in the presence of 20 μM $CaCl_2$ at 45 °C for 2 h; (c) 22 mg was digested with 0.4 mg of thermolysin as above, then 0.4 mg of subtilisin was added, and the incubation was continued for 1 h at 37 °C. Digest a was fractionated by chromatography on Sephadex G-50 in 0.1 M NH_3 solution (Gething & Davidson, 1976). Pooled peptides from the column fractions and digests b and c were purified by paper electrophoresis and chromatography (Davidson, 1970).

Sequencing of Peptides. Sequences were determined by the dansyl–Edman method (Gray, 1972), by carboxypeptidase Y digestion, and by digestion with appropriate proteases followed by purification and analysis of the individual products. Amide groups were assigned by the method of Offord (1966).

Chemical Modification of Native Enzyme. Enyme was reduced overnight in 25 mM dithiothreitol at 4 °C and then chromatographed on Sephadex G-25 equilibrated with buffer M (50 mM *N*-ethylmorpholine/morpholinoethane sulfonate, pH 7.7, 1 mM EDTA) plus 25% glycerol purged with nitrogen. Enzyme (5 μM subunits) was then reacted in the same buffer with 16 μM Nbs_2 in the absence or presence of ligands at 25 °C, and the reaction was followed by the increase in absorbance at 412 nm due to the product, 3-carboxylate 4-nitrothiophenolate (molar extinction coefficient = 14 100 M^{-1} cm^{-1}). At time intervals samples were removed and diluted into buffer M plus 50% glycerol containing 1 mg/mL bovine serum albumin at 0–4 °C for subsequent enzyme assays. Controls lacking Nbs_2 were included to measure inactivation due to nonspecific causes. For iodoacetamide experiments, enzyme (13 μM subunits) in buffer M plus 25% glycerol was reacted with 10 mM iodo[1-^{14}C]acetamide at 25 °C under nitrogen. Samples were removed at time intervals and diluted into buffer M containing 50% glycerol, bovine serum albumin, and 10 mM dithiothreitol. Samples were then assayed for enzymic activity and incorporation of ^{14}C into protein (by scintillation counting and amino acid analysis). Samples of the S-[^{14}C]carboxyamidomethylated enzyme were also used for thermolytic fingerprinting analysis to determine the fate of the ^{14}C label. An incubation at 25 °C for 2 h of enzyme in 5 M guanidine hydrochloride with iodo[1-^{14}C]acetamide provided material for a control fingerprint and identification of the individual peptides by amino acid analysis. The radioactive incorporation into each peptide was determined by cutting out the peptide and counting it in scintillation fluid. The results are expressed as

(^{14}C in peptide)/(total ^{14}C recovered) × moles of ^{14}C reacted per mole of enzyme subunit

Results

Blue Dextran–Sepharose Chromatography. Dye affinity chromatography has proved to be a useful step in the purification of many nucleotide-binding proteins (Turner, 1981). When crude extracts of *E. coli* JP2319 that contain both chorismate mutase/prephenate dehydrogenase, an NAD^+-

Table I: Purification of Chorismate Mutase/Prephenate Dehydrogenase from *E. coli* JP2319

purification step	vol (mL)	total protein (mg)	chorismate mutase act. units	chorismate mutase act. units/mg	prephenate dehydrogenase act. units	prephenate dehydrogenase act. units/mg	recovery (%)	purification factor	mutase: dehydrogenase ratio
(1) cell-free extract[a]	1272	19700	26000	1.32	nd[b]	nd	100	1.00	nd
(2) streptomycin sulfate supernatant	1371	17400	23100	1.33	nd	nd	89	1.01	nd
(3) ammonium sulfate fractionation	102	6740	18700	2.89	nd	nd	72	2.19	nd
(4) chromatography on Blue Dextran–Sepharose	56	136	13300	97.7	11480	84.4	51	74.0	1.16
(5) chromatography on DEAE-Sepharose	20	56	6770	121	5650	101	26	91.7	1.20

[a] Using 225 g of cell paste. [b] Prephenate dehydrogenase activity cannot be determined readily in cell extracts because of the presence of other enzymes that oxidize NADH.

dependent enzyme, and chorismate mutase/prephenate dehydratase, an enzyme not requiring nucleotides, were chromatographed on Blue Dextran–Sepharose at pH 7.1, the former enzyme was bound while the latter was not (Figure 2). Extracts of *E. coli* JP2319 contain significantly higher levels of chorismate mutase/prephenate dehydrogenase than chorismate mutase/prephenate dehydratase because of the *tyrR370* and *trpS378* alleles (Camakaris et al., 1980). Elution of some protein, but not chorismate mutase/prephenate dehydrogenase, was achieved by the addition of 1 mM NAD^+ to the wash buffer, indicating that at pH 7.1 the affinity of this enzyme for the bound dye was stronger than that for the free nucleotide. At pH 8.0 the affinity was weaker and the enzyme could be leached slowly from the adsorbent. Elution at this pH was enhanced by the presence of NAD^+ in the wash buffer and more so by the additional inclusion of 0.5 mM tyrosine. In the absence of NAD^+, tyrosine had little effect on the elution of the enzyme. These conditions of adsorption to, and elution from, Blue Dextran–Sepharose were used as the basis of the purification of the enzyme.

Purification of the Enzyme. Using the method described under Experimental Procedures, we purified chorismate mutase/prephenate dehydrogenase 90-fold from cell extracts of *E. coli* JP2319 (Table I). The enzyme was judged to be homogeneous by SDS–polyacrylamide gel electrophoresis and had specific activities of 121 units/mg of chorismate mutase and 101 units/mg of prephenate dehydrogenase. These figures are well in excess of previously reported specific activities for the enzyme of 19.5 units/mg (Koch et al., 1971a) and 64.5 units/mg of chorismate mutase (SampathKumar & Morrison, 1982) after conversion from 30 to 37 °C units for the latter. Given the documented lability of this enzyme (Koch et al., 1971a), these results indicate that the present procedure yields a highly active and pure enzyme, suitable for structural and kinetic studies. It was noted during the development of the purification procedure that the enzyme was stabilized by the presence of high concentrations of glycerol (25–50% vol/vol) and was markedly unstable in buffers of low ionic strength lacking glycerol.

Structural Properties. The enzyme sedimented as a single symmetrical peak with an apparent $s_{20,w}$ of 4.8 S. Sedimentation equilibrium analysis indicated a native molecular weight of 78000 with no dependence of molecular weight on enzyme concentration in the range 0.2–2 mg/mL. Polyacrylamide gel electrophoresis of the enzyme in the presence of sodium dodecyl sulfate gave a single band of molecular weight 39000, suggesting that the species observed in the sedimentation equilibrium analysis was a dimer composed of identical or closely similar subunits. The amino acid composition (Table II) has therefore been calculated for a subunit molecular weight of 39000. The detection of only 3 unique cysteine-containing sequences and 38 tryptic peptides (discussed below) when twice these numbers were expected per dimer of 78000 provides evidence for the identity of the subunits. From this composition, a value of 0.742 mL/g was calculated for the partial specific volume of the enzyme. The molecular weight and amino acid composition closely match those of SampathKumar & Morrison (1982).

Table II: Amino Acid Compositions of Chorismate Mutase/Prephenate Dehydrogenase and Chorismate Mutase/Prephenate Dehydratase from *E. coli* K12

amino acid	chorismate mutase/prephenate dehydrogenase[a] determined	chorismate mutase/prephenate dehydrogenase[a] nearest integer	chorismate mutase/prephenate dehydratase[b]
Asx	27.4	27	31
Thr	5.5[c]	6	19
Ser	18.5[c]	19	19
Glx	46.6	47	49
Pro	14.6	15	15
Gly	25.7	26	20
Ala	32.1	32	36
Cys	2.4,[d] 2.6[e]	3	4
Val	28.1	28	24
Met	9.3	9	7
Ile	16.3	16	22
Leu	43.9	44	46
Tyr	10.2	10	10
Phe	13.4	13	11
Lys	14.2	14	17
His	8.1	8	13
Arg	23.1	23	20
Trp	5.9[f]	6	2

[a] Calculated for a subunit molecular weight of 39000 from duplicate 24-, 48-, and 72-h hydrolyses. [b] Gething & Davidson (1976). [c] Corrected for destruction by extrapolation to zero time of hydrolysis. [d] By cysteic acid determination after performic acid oxidation. [e] By titration with Nbs_2 in the presence of 4 M guanidine hydrochloride. [f] By the method of Edelhoch (1967).

Kinetic Properties. Double-reciprocal plots for both enzyme activities were linear, indicating hyperbolic saturation curves for chorismate, prephenate, and NAD^+. The K_m for chorismate was 92 μM while the apparent K_m values for prephenate and NAD^+ were 50 μM (at 2 mM NAD^+) and 130 μM (at 0.2 mM prephenate), respectively. The turnover numbers for chorismate mutase and prephenate dehydrogenase were calculated to be 182 and 135 s^{-1}/dimer, respectively.

S-[^{14}C]Carboxymethylation of Chorismate Mutase/Prephenate Dehydrogenase. S-[^{14}C]Carboxymethylation of reduced and denatured chorismate mutase/prephenate dehydrogenase with iodo[2-^{14}C]acetate resulted in the incorporation of 3.1 mol of ^{14}C/mol of enzyme subunit. Amino acid analysis of the modified enzyme revealed one extra peak,

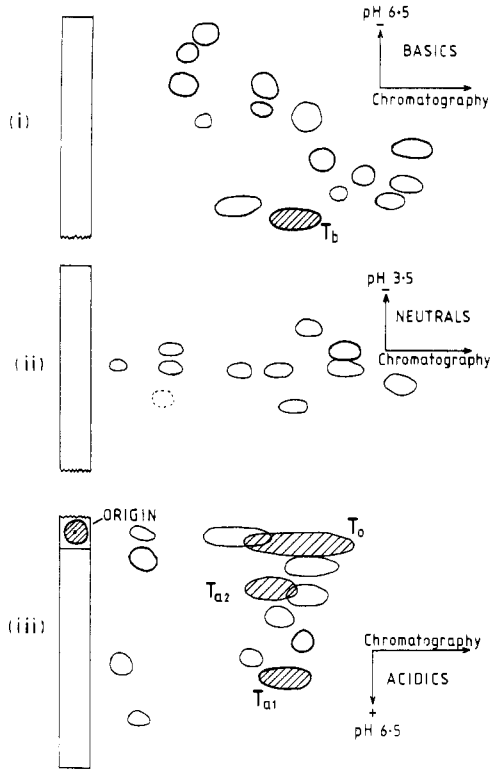

FIGURE 3: Tracings of tryptic fingerprints of S-[^{14}C]carboxymethylated chorismate mutase/prephenate dehydrogenase. After electrophoresis at pH 6.5, the (i) basic and (iii) acidic peptides were chromatographed at right angles by using butan-1-ol/acetic acid/water/pyridine (30/6/24/20 by volume) as the solvent, while the (ii) neutral peptides were electrophoresed at pH 3.5 and then chromatographed. The thickness of the outline indicates the relative intensity of staining with fluorescamine, while the shading represents ^{14}C-labeled peptides.

corresponding to Cmc, indicating that under the experimental conditions only the cysteine residues of the enzyme reacted. This stoichiometry of three cysteine residues per subunit agrees with the earlier determination (Table II).

Purification and Sequencing of Tryptic Peptides. Tryptic fingerprints of the S-[^{14}C]carboxymethylated enzyme revealed four soluble radioactive peptides and additional insoluble or "core"material at the origin (Figure 3). A total of about 38 peptides was observed after staining with fluorescamine, close to the total number of lysine plus arginine residues determined per subunit of enzyme (Table II) and a further indication of the identify of the primary structure of the enzyme subunits.

Large-scale purification of the radioactive peptides by G-50 chromatography and paper electrophoresis yielded four radioactive peptides (Table III), one of which, To, was large, did not migrate on paper electrophoresis and did not give a satisfactory end group by dansyl analysis. Amino acid analysis and redigestion with either chymotrypsin or subtilisin also indicated that it was partially digested material. The amino acid sequences of peptides Tb and Ta1 were determined satisfactorily, while sequencing of peptide Ta2 by the dansyl–Edman method became blocked after the third valine residue (Figure 4).

Purification and Sequencing of Thermolytic Peptides. In order to complete the determination of the amino acid sequences around the cysteine residues, thermolysin was used both alone and in combination with subtilisin to generate additional peptides for sequencing (Table III and Figure 4). Only three radioactive spots were observed on thermolytic fingerprints, none of which was located at the origin. A major difficulty experienced was with the thermolytic peptide La2, which trailed when electrophoresed at pH 3.5 and appeared by both amino acid analysis and the dansyl–Edman procedure to be a mixture of peptides differing both in the number of valine residues at the N terminus and in the presence or absence of tyrosine at the C terminus. As was observed with peptide Ta2 (see above) the dansyl–Edman procedure was blocked after the third valine residue. This problem was solved by the isolation of a thermolytic/subtilitic peptide, LSa2, which gave a complete amino acid sequence with no indication of blocking (Figure 4). The identity of the blocking group was not determined but it may have been an oxidation product of a labile residue, or alternatively Cmc (or its sulfone) failing to react with dansyl chloride and phenyl isothiocyanate after one round of Edman degradation. The position in the sequence is indicated with X (Figure 4).

As a consequence of the observation of a maximum of three unique cysteine residues in both of the thermolytic digests, it

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I.   Tb        Thr-Leu-Cmc-Pro-Ser-Leu-Arg-Pro-Val-Val-Ile-Val-Gly-Gly-Gly-Gly-Gln(Met,Gly)Arg
   Thermolytic <-----------------> <---------> <--------------------------------> <--------->
  peptides of Tb
    La,LSa          Leu-Cmc-Pro-Ser

   Deduced      Thr-Leu-Cys-Pro-Ser-Leu-Arg-Pro-Val-Val-Ile-Val-Gly-Gly-Gly-Gly-Gln-Met-Gly-Arg
   sequence

II.  Ta1                                                  Asp-Cmc-Ile-Leu-Val-Asp-Leu(Ala,Ser,Val,Lys)
   Thermolytic                                            <-----> <-----> <-----> <---------> <-----
  peptides of Ta1
    Ln,LSn      Val-Ile-Gly-Lys-Leu-Pro-Pro-Leu-Pro-Lys-Asp-Cmc
   Tryptic      <-------------> <--------------------> <----->
  peptides of Ln

   Deduced      Val-Ile-Gly-Lys-Leu-Pro-Pro-Leu-Pro-Lys-Asp-Cys-Ile-Leu-Val-Asp-Leu-Ala-Ser-Val-Lys
   sequence

III. Ta2        Gln-Val-Val-Val( X ,Cmc,Asp,Gly,Arg)
     La2            Val-Val-Val( X ,Cmc,Asp,Gly,Arg,Lys,Pro,Glu)Ala-Tyr
     LSa2                        Cmc,Asp,Gly-Arg-Lys-Pro-Glu-Ala-Tyr
   Tryptic                       <-------------> <----------------->
  peptides of LSa2

   Deduced      Gln-Val-Val-Val- X -Cys-Asp-Gly-Arg-Lys-Pro-Glu-Ala-Tyr
   sequence
```

FIGURE 4: Deduced sequences surrounding the three unique cysteines (I, II, III) of chorismate mutase/prephenate dehydrogenase. Peptides Tb, La1, LSa1, Ta1, Ln, LSn, Ta2, and LSa2 containing *S*-([^{14}C]-carboxymethyl)cysteine were purified as described in Table III. Their sequences were determined by the dansyl–Edman method, carboxypeptidase Y digestion, and amino acid analysis. Residues shown in parentheses were detected by amino acid analysis. Sequence assignments were confirmed by thermolytic digestion (of Tb and Ta1) or tryptic digestion (of Ln and LSa2), fractionation of the individual products, and sequence analysis. X represents an uncertain residue (see the text).

Table III: Analysis of Purified Peptides of S-[^{14}C]Carboxymethylated Chorismate Mutase/Prephenate Dehydrogenase[a]

amino acid, details	tryptic peptides				thermolytic peptides			thermolytic/ subtilitic peptides		
	Tb	Ta1	Ta2	To	La1	Ln	La2	LSa1	LSn	LSa2
^{14}C	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lys		1.1		1.5		1.8	0.9		1.7	0.6
His				0.6						
Arg	2.0		1.0	2.7			0.8			0.9
Cmc	0.3	0.2	+	0.5	+	+	+	0.6	0.2	0.5
Asx		2.1	1.3	3.0		1.3	1.0		1.4	1.1
Thr	0.8			0.9						
Ser	0.9	0.9		1.5	0.9			1.0		
Glx	1.3		1.1	10.0			1.1			1.2
Pro	2.0			2.4	1.0	3.5	0.9	1.0	2.9	0.9
Gly	5.0		1.4	3.8		1.3	1.1		1.4	1.0
Ala		1.0		5.0			1.1			1.2
Val	3.4	2.2	2.6	4.7		0.6	1.0		1.0	
Met	1.1			1.1						
Ile	0.9	0.9		3.3		0.7			0.7	
Leu	2.2	2.1		4.2	1.0	2.3		1.0	2.2	
Tyr				1.8			0.7			1.0
Phe				2.2						
Trp[b]				+						
purification	G, 6, 2	G, 6, B	G, 6, O, 2	G, 6, B	6, B, O, 2	6, 3, O, 2	6, O, B	6, B, 2	6, B, 2	6, B, 2
mobility, pH 6.5	+0.19	−0.50	−0.28	origin	−0.46	0	−0.20	−0.46	0	−0.23
amount (nmol)	350	96	51	212	20	17	18	49	34	48
yield (%)	14	4	2	8	8	7	7	9	6	9
N terminus	Thr	Asx	Glx	nd	Leu	Val	Val	Leu	Val	Cmc

[a] The amounts of enzyme used for each digest were as follows: trypsin, 98 mg; thermolysin, 10 mg; thermolysin/subtilisin, 22 mg. The nomenclature for the peptide names are as follows: T, T, tryptic peptide; L, thermolytic peptide; LS, thermolytic/subtilitic peptide; o, origin; a, acidic at pH 6.5; b, basic at pH 6.5; n, neutral at pH 6.5. Analyses were done on 24-h hydrolysates except for Tb, La2, and Ln, which were 48-h hydrolysates. The composition for La2 is of one of the major species of this group of peptides (see the text). The symbols mean the following: (+), detected qualitatively; nd, not determined. The key to the purification steps is as follows: G, chromatography on Sephadex G-50; 6, 3, and 2, paper electrophoresis at pH 6.5, 3.5, and 2.1, respectively; B, paper chromatography; O, oxidation with performic acid. [b] Determined by Ehrlich's reagent.

was concluded that this is the total number of unique cysteine residues in the enzyme. The presence of two additional radioactive spots in the tryptic fingerprint (Figure 3: To and the origin material) is ascribed to incompleteness in the tryptic digestion arising as a consequence of the insolubility of partial digestion products. The deduced amino acid sequences around these three cysteine residues (numbered I to III) are shown in Figure 4.

Modification of the Enzyme with Nbs_2. Preliminary experiments showed that purified chorismate mutase/prephenate dehydrogenase was unstable in sulfhydryl-free buffers at 25 °C under the conditions required for chemical modification. Inclusion of 25% glycerol in the buffer stabilized the enzyme considerably so that loss of activity in the absence of modifying reagents was decreased to less than 10% during the reaction time of 1–2 h. Reaction of enzyme with Nbs_2 in denaturing conditions (5 M guanidine hydrochloride) was complete in 10 min. The number of moles of Nbs_2 reacting per mole of enzyme subunit was found repeatedly to be 2.6 to 2.7 by using enzyme that had been pretreated with dithiothreitol to give a maximal sulfhydryl content.

When native enzyme was reacted with a slight molar excess of Nbs_2 over protein sulfhydryl groups, an average of two sulfhydryl groups were modified (Figure 5A). This reaction was characterized by a fast reaction of 1 mol of sulfhydryl group/mol of enzyme subunit and a slower reaction of a second mole. A rapid loss of approximately 70% of both enzyme activities was observed, concomitant with the initial reaction with 0.7 sulfhydryl group per subunit. Inactivation of the remaining activity took place at a much slower rate and approached 100% as the total reaction approached 2 mol of sulfhydryl group reacted/mol of enzyme subunit. The third cysteine residue either reacted very slowly or was inaccessible to Nbs_2. When 10 mM 2-mercaptoethanol was added to the reaction mixture after 1 h and the incubation was extended for a further hour, recovery of 80% of both initial activities was observed, indicating that the inactivation by Nbs_2 was reversible. These results suggest that the initial reaction of Nbs_2 was with a fast-reacting cysteine residue that may be essential for both enzyme activities. It is unclear why the rates of reaction and inactivation were slower beyond 0.7 group modified/subunit but may reflect reaction at the fast-reacting group on one subunit affecting the reactivity of the equivalent group on the neighboring subunit. Second-order plots of these data showed no obvious linear sections indicative of two independently reacting groups (not shown), indicating that the reactions of these groups were either not second order or independent. Treatment of the data by the method of Ray and Koshland (1961) for first-order analysis was not possible since Nbs_2 was not in a large excess.

Because of the reversibility of the Nbs_2 reaction, the identity of the modified cysteine residues could not be further investigated by fingerprinting studies.

Protection of the Enzyme from Nbs_2 Modification. The effects of various ligands on the rate of modification by Nbs_2 were determined by incubating the enzyme with ligand prior to the addition of reagent. Prephenate (1 mM) effectively abolished the reaction with 1 mol of sulfhydryl group/mol of subunit and protected the two enzymic activities (Figure 5B). By contrast, 0.5 mM tyrosine or (4-hydroxyphenyl)pyruvate, the product of the prephenate dehydrogenase reaction, had no significant effect on the rate of reaction, while 1 mM NAD^+ or NADH were only partially effective in reducing the reaction by Nbs_2 (not shown). Tyrosine enhanced the protective effect of NAD^+ such that this combination was the most effective examined for reducing the rate of reaction and protecting the enzymic activities (Figure 5C). This implies that the presence of prephenate or NAD^+ inhibits modification of a very reactive sulfhydryl group that may be essential for enzyme activity. Since the extent of reaction with Nbs_2 exceeded the extent of

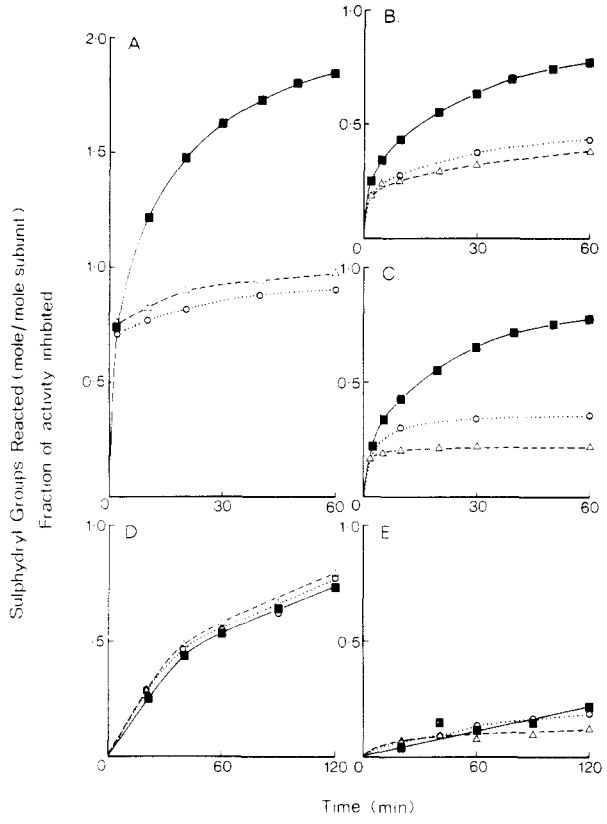

FIGURE 5: Chemical modification of the sulfhydryls of chorismate mutase/prephenate dehydrogenase. Reduced enzyme (5 μM subunits) was reacted with 16 μM Nbs_2 at 25 °C in (A) the absence of ligands, (B) the presence of 1.0 mM prephenate, and (C) the presence of 1.0 mM NAD^+ plus 0.5 mM tyrosine. Reduced enzyme (13 μM subunits) was reacted with 10 mM iodo[1-^{14}C]acetamide at 25 °C under nitrogen in (D) the absence of added ligands and (E) the presence of 1.0 mM NAD^+ plus 0.5 mM tyrosine. Sulfhydryl groups reacted (■); inhibition of chorismate mutase activity (○); inhibition of prephenate dehydrogenase activity (△).

inhibition in the presence of these ligands (Figure 5B,C), it can also be concluded that under these conditions reaction occurs, at least partly, with a sulfhydryl group that is not essential for enzyme activity.

Modification of the Enzyme with Iodo[1-^{14}C]acetamide. Both iodoacetate and iodoacetamide reacted with the native enzyme, causing loss of the two activities. The reaction with iodoacetamide was the more rapid of the two, suggesting that a negative charge on the enzyme hinders the approach of iodoacetate. The inactivation of both activities during the reaction of native enzyme with 10 mM iodo[1-^{14}C]acetamide was parallel with and strictly proportional to the extent of reaction (Figure 5D). Furthermore, since only 1 mol of sulfhydryl group reacted/mol of enzyme subunit, it is possible that only one cysteine residue was available for modification. The effect of NAD^+ plus tyrosine was quite striking in almost completely protecting the enzyme from reaction and inactivation (Figure 5E), but by contrast to the Nbs_2 results incorporation did not exceed inhibition. Replotting this data as the logarithm of activity or unmodified groups remaining vs. time (Ray & Koshland, 1961) revealed curved plots, so that the reaction kinetics were not pseudo first order (not shown). The rate of reaction slowed beyond 0.4–0.5 group modified per subunit.

Identification of the Modified Cysteines. Peptide fingerprinting was carried out to determine if the carboxamidomethylation reaction was specific for a single cysteine residue. When chorismate mutase/prephenate dehydrogenase was reacted for 2 h at 25 °C with iodo[1-^{14}C]acetamide in 5 M guanidine hydrochloride, autoradiographs of thermolytic fingerprints revealed three major radioactive peptides, each of which was shown to contain one of the three unique cysteine residues of the enzyme. The identity of the cysteine residue in each peptide was established by amino acid analysis, and the relative incorporation of ^{14}C into the different cysteine residues was determined (Table IV). Incorporation into cysteine II was relatively low, suggesting that the sulfhydryl group of this residue is buried deeply in a structure that is stable even in the presence of 5 M guanidine hydrochloride, or that it is partially oxidized, despite the precautions taken to ensure maximal reduction of the enzyme before reaction with iodo[1-^{14}C]acetamide (see Experimental Procedures).

Table IV: Incorporation of ^{14}C into the Cysteine Residues of Chorismate Mutase/Prephenate Dehydrogenase by Reaction with Iodo[1-^{14}C]acetamide[a]

cystiene residue	relative incorporation of ^{14}C per peptide (mol/mol of subunit)		
	enzyme alone	enzyme + 1 mM NAD^+ + 0.5 mM tyrosine	enzyme + 5 M Gdn-HCl
I	0.11	0.05	0.91
II	0.03	0.02	0.37
III	0.58	0.15	1.11
origin	0.04	0.02	0.01
total	0.75	0.25	2.40

[a] Enzyme was reacted for 2 h at 25 °C under the conditions described under Experimental Procedures with additions as indicated. Samples were then used to prepare thermolytic fingerprints. Three radioactive peptides were detected as well as a small amount of radioactivity at the origin. The identity of the peptides was established by amino acid analysis of purified *S*-[([^{14}C]carboxamido)methyl]cysteine-containing peptides prepared in a parallel fashion.

When the native enzyme was reacted with iodo[1-^{14}C]-acetamide and thermolytic fingerprints were again prepared, most of the ^{14}C appeared in the peptide containing cysteine III, although a small amount of incorporation into the peptide of cysteine I was detected (Table IV). This indicates that the three cysteine residues have differing reactivities in the native enzyme, with cysteine III being the most reactive and cysteine II the least. When considered in conjunction with the inactivation kinetics, it also suggests that cysteine III may be essential for both enzymic activities. In the presence of NAD^+ plus tyrosine, the reaction of iodoacetamide with cysteine III was decreased significantly (by 0.43 mol/mol of enzyme subunit) and the labeling of cysteine I was lowered only slightly (by 0.06 mol/mol of enzyme subunit), thereby adding support to the idea that modification of cysteine III leads directly to loss of the two enzymic activities.

Discussion

Purification and Properties of Chorismate Mutase/Prephenate Dehydrogenase. The purification procedure described above yields enzyme that is homogeneous by polyacrylamide gel electrophoresis and sediments as a single peak in the ultracentrifuge. The specific activity of this enzyme is approximately 6 times higher than that reported in the first purification of chorismate mutase/prephenate dehydrogenase (Koch et al., 1971a) and twice that reported recently by SampathKumar and Morrison (1982). Attainment of this higher specific activity could be due to a number of reasons, e.g., the avoidance of dialysis during the purification, the use of glycerol, and the fewer chromatography steps. The fact that chromatography on Blue Dextran–Sepharose yields a significantly better purification factor than does chromatography on Sepharose–AMP (SampathKumar & Morrison,

Table V: Comparison of Properties of Chorismate Mutase/Prephenate Dehyrogenase and Chorismate Mutase/Prephenate Dehydratase from *E. coli* K12

property	chorismate mutase/ prephenate dehydrogenase	chorismate mutase/ prephenate dehydratase
subunit		
size	39 000–42 000[a,b]	40 000[d]
arrangement	homodimer[a,b]	homodimer[d]
self-association		
species formed	tetramers[c]	tetramers and higher polymers[e]
conditions	high [tyrosine], [NAD^+][c]	high [phenylalanine], pH, ionic strength[e]
intracellular concn (wild-type cells) (μM subunits)	~0.5	~0.5[f]
K_m (μM)		
chorismate	92	45[g]
prephenate	50	1000[g]
turnover number (per dimer) (s^{-1})		
chorismate mutase	182	225[f]
prephenate dehydrogenase	132	
prephenate dehydratase		136[f]
end-product inhibition		
inhibitor	tyrosine (requiring NAD^+)[c]	phenylalanine[g]
type		
chorismate mutase	partial sigmoidal[c]	partial competitive[g]
prephenate dehydrogenase	sigmoidal[c]	
prephenate dehydratase		sigmoidal[g]
active sites		
sulfhydryl modification	both activities lost	prephenate dehydratase activity lost[h]
interrelationship	interacting	independent

[a] Koch et al. (1971a). [b] SampathKumar & Morrison (1982). [c] Hudson et al. (1983). [d] Davidson et al. (1972). [e] Baldwin et al. (1981). [f] Calculated from the specific activities obtained by K. H. C. Ma and B. E. Davidson (unpublished results), which were higher than those reported previously. [g] Dopheide et al. (1972). [h] Gething & Davidson (1977a,b).

1982) probably eliminates the need for more than one subsequent purification steps in our method. The use of a strain of *E. coli* carrying appropriate mutations in regulatory genes is of considerable advantage in obtaining a good yield of highly active enzyme. We have also used this procedure successfully to purify chorismate mutase/prephenate dehydrogenase in high yields from strains of *E. coli* containing a multicopy plasmid, pMU1000, that carries *tyrA*, the structural gene for chorismate mutase/prephenate dehydrogenase (Hudson et al., 1983).

Chorismate mutase/prephenate dehydrogenase bound to Blue Detran–Sepharose was partially eluted by NAD^+ but was eluted more satisfactorily by a combination of both NAD^+ and tyrosine (tyrosine alone caused no elution). This observation can be explained by reference to the results of binding experiments done in the air-driven ultracentrifuge with pure chorismate mutase/prephenate dehydrogenase, which showed that the binding of NAD^+ and tyrosine to the enzyme is synergistic (Hudson et al., 1983). It seems likely that the enzyme binds to the dye through the NAD^+-binding site and that elution by NAD^+ is enhanced by tyrosine because of these cooperative interactions. Alternatively, the dye might bind elsewhere on the enzyme to a site that is disrupted when NAD^+ is bound to the NAD^+-binding site.

Investigation of the kinetic properties of the purified enzyme revealed hyperbolic saturation curves for chorismate, prephenate, and NAD^+, with no indication of nonlinearity in any of the double-reciprocal plots. In this respect our results differ from those of Koch et al. (1971a) who found nonlinear double-reciprocal plots, but agree closely with those reported recently by SampathKumar and Morrison (1982). The K_m determined for chorismate was 92 μM, considerably lower than that reported by Koch et al., but similar to the K_m for chorismate mutase/prephenate dehydratase (Dopheide et al., 1972). In vivo, both chorismate mutase/prephenate dehydratase and chorismate mutase/prephenate dehydrogenase compete for chorismate with anthranilate synthetase/anthranilate 5′-phosphoribosyl-1-pyrophosphate phosphoribosyltransferase, which has a K_m of only 1.2 μM for chorismate (Baker & Crawford, 1966). The physiological significance of this marked difference in K_m values is not obvious although it is clear that the tryptophan pathway has the potential to compete most effectively with the phenylalanine and tyrosine pathways for chorismate when the concentration of this metabolite is low.

Using a viable cell count of 2×10^{12} cells (4 g wet weight) of JP2319 per L at harvest, a specific activity of pure enzyme of 121 units/mg and an *E. coli* cell volume of 1 fL (Luria, 1960), one can calculate the average intracellular concentration of chorismate mutase/prephenate dehydrogenase in JP2319 to be approximately 40 μM subunits. In wild-type *E. coli* grown in the absence of aromatic end products, the concentration would be 0.5 μM subunits (or 300 subunits per cell) and the total turnover numbers per cell would be 3×10^4 and 2×10^4 s^{-1} for the chorismate mutase and prephenate dehydrogenase activities, respectively. Wild-type cells also contain approximately the same level of chorismate mutase/prephenate dehydratase with similar turnover numbers for its two activities (Table V), so that the potentials of the cell for phenylalanine and tyrosine biosynthesis are balanced under these conditions.

Reactive Cysteine and Relationship between Active Sites. Chorismate mutase/prephenate dehydrogenase contains three unique cysteines per subunit (Figure 4). Modification of native enzyme with iodoacetamide (or, by analogy, Nbs_2) revealed a difference in reactivity of these groups. The sulfhydryl of cysteine III was highly reactive while those of cysteines I and II reacted more slowly or not at all. Reaction of cysteine III was associated with a parallel loss of both activities, and protection of this residue by NAD^+ (in the presence of tyrosine, but not tyrosine alone) or prephenate also protected the enzymic activities. We cannot determine from the data if this residue is truly essential for the function of the enzyme; however, it is likely that this residue is located close to the active sites (in particular, the prephenate and NAD^+ binding sites) and its reactivity could account for some of the lability of the enzyme in vitro. The parallel loss of both mutase and

dehydrogenase activities suggest a close spatial relationship between the sites for these two functions.

The relationship between the two activities is of considerable interest to determine the effects of bifunctionality on catalysis. The two activities of the enzyme from *E. coli* or *A. aerogenes* appear to be interdependent. NAD^+ activates chorismate mutase activity, and conversely, chorismate stimulates prephenate dehydrogenase activity (Heyde & Morrison, 1978). Prephenate formed at the chorismate mutase site is more accessible for subsequent conversion to (4-hydroxyphenyl)-pyruvate than would be expected if there were an intervening dissociation and reassociation (Koch et al., 1972; Heyde, 1979). It is possible that the two activities are catalyzed at a single site (Andrews & Heyde, 1979), and such a proposal is supported by the observation of parallel inactivation of both activities during chemical modification of the enzyme (Koch et al., 1972; Heyde, 1979; this paper). Evidence against this proposal is that tyrosine preferentially inhibits the prephenate dehydrogenase activity (Hudson et al., 1983), specific antisera raised against the enzyme cause preferential inhibition of the chorismate mutase activity (G. S. Hudson and B. E. Davidson, unpublished results), and a mutant enzyme lacking prephenate dehydrogenase activity but retaining chorismate mutase activity has been isolated (Rood et al., 1982). Hence, the two activities of chorismate mutase/prephenate dehydrogenase appear to be catalyzed at closely situated and interacting active sites.

Comparison with Chorismate Mutase/Prephenate Dehydratase. Chorismate mutase/prephenate dehydrogenase and chorismate mutase/prephenate dehydratase are bifunctional and have similar functions and substrates (Figure 1). A comparison of their known properties (Table V) and amino acid compositions (Table II) reveals other similarities, both structural and kinetic. The enzymes are each composed of subunits of molecular weight 40 000 of similar amino acid composition. Both enzymes are subject to end-product inhibition, chorismate mutase/prephenate dehydrogenase by tyrosine in the presence of NAD^+ (Hudson et al., 1983) and chorismate mutase/prephenate dehydratase by phenylalanine (Dopheide et al., 1972). In both cases the second activity, involving prephenate, is the more sensitive to inhibition, while the chorismate mutase activity is only partially inhibited by the end product. Furthermore, the mechanism of end-product inhibition of each enzyme involves polymerization of enzyme dimers to species of higher molecular weight and lower enzymic activity (Baldwin et al., 1981; Hudson et al., 1983). The major difference between the two enzymes, apart from their K_m values for prephenate, lies in the relationship between the two active sites. As discussed above, the active sites of chorismate mutase/prephenate dehydrogenase are considered to be interacting. By contrast, many lines of evidence suggest that the two activities of chorismate mutase/prephenate dehydratase are catalyzed at distinct and independent sites; for example, there is differential inhibition of the two activities by chemical modifying reagents (Gething & Davidson, 1977a,b), phenylalanine (Dopheide et al., 1972), and substrate analogues (Baldwin & Davidson, 1983). In addition, prephenate is not channeled between the two active sites (Duggleby et al., 1978) and mutants have been isolated that are defective in either the chorismate mutase or prephenate dehydratase activity (Baldwin & Davidson, 1981). These differences aside, it is possible that all or part of the genes for these two enzymes have evolved through duplication of an ancestral gene. It is known that antisera raised against one enzyme can inhibit but not immunoprecipitate the other enzyme, implying some limited homology between the two enzymes (G. S. Hudson and B. E. Davidson, unpublished results).

Both enzymes possess highly reactive sulfhydryl groups. Chorismate mutase/prephenate dehydratase has four cysteines per subunit (Gething & Davidson, 1976), and modification of the most reactive residues with *N*-ethylmaleimide caused inhibition of the prephenate dehydratase activity only (Gething & Davidson, 1977b; Ma & Davidson, 1984). However, alignment of the sequences of Figure 4 with those of the cysteine-containing peptides and other tryptic peptides from chorismate mutase/prephenate dehydratase (Gething & Davidson, 1976; Baldwin & Davidson, 1979) showed no significant homologies. This suggests that the proteins are not closely related.

Acknowledgments

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Registry No. Cysteine, 52-90-4; chorismic acid, 617-12-9; prephenic acid, 126-49-8.

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Comparative Phosphorescence and Optically Detected Magnetic Resonance Studies of Pig and Yeast Glyceraldehyde-3-phosphate Dehydrogenase†

Janice M. Davis and August H. Maki*

ABSTRACT: A comparative optically detected magnetic resonance (ODMR) investigation has been made of the tryptophan (Trp) residues of glyceraldehyde-3-phosphate dehydrogenase (GAPD) from pig and yeast. We find that pig GAPD emits phosphorescence from only two of the three distinct Trp sites, while yeast GAPD exhibits resolved 0,0-bands from all three Trps. Heavy atom effects observed in the $CH_3Hg(II)$–sulfhydryl complex of pig GAPD resemble closely those reported earlier for the analogous rabbit GAPD–$CH_3Hg(II)$ complex. Trp-310, with a 0,0-band at 416 nm, undergoes a selective heavy atom perturbation as a result of $CH_3Hg(II)$ binding to the nearby Cys-281. The 416-nm peak in yeast GAPD is assigned to Trp-310 on the basis of ODMR, but no heavy atom effect of $CH_3Hg(II)$–sulfhydryl complexing is observed because of the absence of Cys-281 in yeast, thus supporting this assignment. The 406-nm 0,0-bands of pig and rabbit GAPD and the 409-nm band of yeast GAPD are assigned to Trp-193, located in a subunit contact region. This residue is solvent exposed in the yeast enzyme but appears to be buried in a polar environment in the mammalian GAPD. These differences may be related to variations in subunit cooperativity between species. Trp-84 appears to be quenched in pig and rabbit GAPD, most likely by His-108. In yeast GAPD, on the other hand, Trp-84 is not quenched, probably because His-108 is further removed. The Trp-84 0,0-band of the yeast enzyme peaks at 420 nm, making it the most red-shifted Trp origin reported thus far. The influence of local perturbations on the triplet-state properties of specific Trp sites in these enzymes is discussed.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD,[1] EC 1.2.1.12) is a glycolytic enzyme, catalyzing the reaction

$$\text{glyceraldehyde 3-phosphate} + NAD^+ + P_i = \text{1,3-diphosphoglycerate} + NADH + H^+$$

It has a molecular weight of 144 000 and consists of four 36 000-dalton monomers with identical sequences. The sequences of the lobster, pig, and yeast enzymes are known (Olsen et al., 1975), as well as those of human GAPD (Nowak et al., 1981) and GAPD from chicken heart (Domdey et al., 1983) (93%) and from two thermophilic bacteria (Walker et al., 1980; Hocking & Harris, 1980). The sequence is not known for rabbit GAPD (which along with the yeast enzyme is commercially available). X-ray studies have been done on lobster GAPD (Moras et al., 1975) and of the *Bacillus stearothermophilus* enzyme (Biesecker et al., 1977). Although the monomer sequences are the same for each species, small differences may occur in their conformations. The lobster GAPD appears to be an asymmetric dimer of dimers, with identical so-called "red" and "yellow" subunits as well as identical "blue" and "green" subunits (Moras et al., 1975). The sequences of GAPD's tend to be highly conserved (Domdey

† From the Department of Chemistry, University of California, Davis, California 95616. *Received March 27, 1984; revised manuscript received July 3, 1984.* This research was supported by a grant, ES-02662, from the National Institutes of Health.

[1] Abbreviations: ODMR, optical detection of magnetic resonance; GAPD, glyceraldehyde-3-phosphate dehydrogenase; zfs, zero-field splittings; AM-PMDR, amplitude-modulated phosphorescence/microwave double resonance; NAD, nicotinamide adenine dinucleotide.

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