Substrate-Decreased Modification by Diethyl Pyrocarbonate of Two Histidines in Isocitrate Lyase from *Escherichia coli*†

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ABSTRACT: The inactivation of tetrameric 188-kDa isocitrate lyase from *Escherichia coli* at pH 6.8 (37 °C) by diethyl pyrocarbonate, exhibiting saturation kinetics, is accompanied by modification of histidine residues 266 and 306. Substrates isocitrate, glyoxylate, or glyoxylate plus succinate protect the enzyme from inactivation, but succinate alone does not. Removal of the carbethoxy groups from inactivated enzyme by treatment with hydroxylamine restores activity of isocitrate lyase. The present results suggest that the group-specific modifying reagent diethyl pyrocarboate may be generally useful in determining the position of active site histidine residues in enzymes.

When microbes grow on two-carbon compounds, isocitrate lyase [*threo*-D_s-(+)-isocitrate glyoxylate lyase; EC 4.1.3.1], the first unique enzyme of the anaplerotic glyoxylate bypass, catalyzes the reversible cleavage of isocitrate into glyoxylate and succinate. The gene for this enzyme in *Escherichia coli* has been cloned, sequenced, and overexpressed (Matsuoka & McFadden, 1988). The results of studies by Ko and McFadden (1990a) with 3-bromopyruvate suggest that cysteine 195 is at the active site. This residue has also been labeled by using iodoacetate (Nimmo et al., 1989).

In spite of the importance of isocitrate lyase in microbial assimilation of two-carbon substrates, germination of fat-rich seeds, and embryogenesis in nematodes (Vanni et al., 1990), little is known about its catalytic action. We now describe the use of diethyl pyrocarbonate to modify this enzyme. Conditions are described which enable isolation and sequencing of resultant carbethoxylated histidine residues. Residues 266 and 306 appear to be in the active site domain.

Experimental Procedures

Chemicals. The following reagents were obtained from Sigma Chemical Co.: D_s-isocitrate trisodium salt (provided as DL-isocitrate); glyoxylic acid sodium salt monohydrate; diethyl pyrocarbonate (DEP);[1] glutathione; itaconic acid; 3-(*N*-morpholino)propanesulfonic acid sodium salt (MOPS); phenylhydrazine hydrochloride; *N*-ethylmaleimide; and succinic acid. Sephadex G-50 was obtained from Pharmacia. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and potassium phosphate monobasic (Ultrex Ultrapure Reagent) were supplied by J. T. Baker. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich Chemical Co. [^{3}H]Diethyl pyrocarbonate ([^{3}H]DEP), $CH_3CHTOC(O)OC(O)CHTCH_3$, was synthesized by Igarashi et al. (1985) and stored at −70 °C in sealed glass ampules. All other reagents were of the highest grade obtainable.

Isocitrate Lyase. Overproduction of isocitrate lyase was obtained in a culture of *E. coli* JE 10 which had been transformed with the pICL-1 plasmid (Matsuoka & McFadden, 1988). The enzyme was purified to homogeneity according to the procedure of Conder et al. (1988). It was stored at −70 °C in an isolation buffer consisting of 50 mM triethanolamine, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.3.

Activation of Enzyme and Exchange of Buffers. Isocitrate lyase in the isolation buffer was first reduced with 5 mM dithioreitol at 37 °C for 5 min. To change buffers, the enzyme preparation was then sedimented through a Sephadex G-50 column at 25 °C (Penefsky, 1977) that had previously been equilibrated with PME buffer (50 mM potassium phosphate buffer containing 5 mM $MgCl_2$ and 1 mM EDTA, pH 6.6) or MME buffer (50 mM MOPS buffer containing 5 mM $MgCl_2$ and 1 mM EDTA, pH 7.3). The PME buffer was used for inactivation studies with diethyl pyrocarbonate. MME buffer was used for all other procedures unless otherwise indicated.

Isocitrate Lyase Assay. The discontinuous method of assay of isocitrate lyase in the direction of the cleavage of isocitrate, as described by Jameel et al. (1984), was used except that the temperature of the incubation mixture was 37 °C and the pH was 7.3. This method entails the spectrophotometric measurement of the cleavage product, glyoxylate, after its reaction with phenylhydrazine and subsequent oxidation of the resulting phenylhydrazine with potassium ferricyanide. The absorbance of the resulting chromophore was measured at 535 nm by using a Varian DMS 80 UV/vis spectrophotometer. The formation of glyoxylate was linear with time.

Protein Determination. Protein concentrations were determined spectrophotometrically by using Pierce's Micro BCA (bioinchoninic acid) protein assay reagent (see Pierce Handbook and General Catalog, p 212) with lysozyme as a standard.

Saturation Kinetics. Inactivation of isocitrate lyase was accomplished at 37 °C with varying concentrations of absolute ethanol solubilized DEP diluted into MME buffer, pH 6.8. The reactions were quenched with 16 mM histidine (pH 6.8). The ethanol concentration did not exceed 5% by volume and was found to have no effect on the activity and stability of the enzyme during the incubation times. The k_{obs} value (*pseudo*-first-order rate constant) for each concentration of DEP was obtained from the slope of the plot of ln [relative isocitrate lyase] vs incubation time.

Substrate Protection. Isocitrate lyase was preincubated with its substrates individually in MME buffer at 37 °C, pH 6.8, for 5 min prior to incubation with 0.5 mM DEP for 3 min.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; DEP, diethyl pyrocarbonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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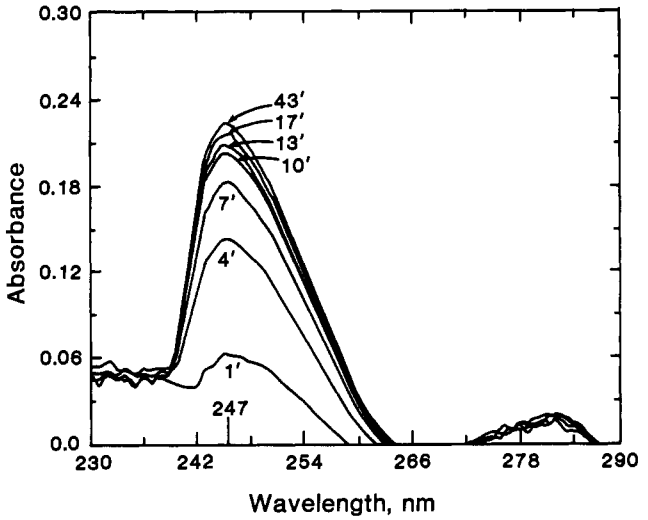

FIGURE 1: UV difference spectra showing the effect of time of incubation of isocitrate lyase with DEP. This spectroscopic study was done at 30 °C in buffer, pH 6.5, starting with DEP at 0.5 mM and isocitrate lyase at 25 μM subunits; incubation times are shown. The absorbance shown has been computer-corrected for blank isocitrate lyase in the range of 230–290 nm for each time of incubation. See Experimental Procedures for other details.

Reactions were quenched with 16 mM histidine (pH 6.8); the mixtures were gel filtered by centrifugal sedimentation through columns of Sephadex G-50 that had previously been equilibrated with MME buffer. Following this treatment the residual isocitrate lyase activity of each sample was determined.

UV Difference Spectra. Spectroscopic studies were performed at 30 °C in 100 mM phosphate containing 10 mM $MgCl_2$ and 2 mM EDTA, pH 6.5, starting with DEP at 0.5 mM and isocitrate lyase at 25 μM in subunits. The recorded absorbance was computer-corrected for the blank (enzyme in buffer without DEP) in the range of 230–290 nm for each time of incubation (see Figure 1). The stoichiometry of the formation of the *N*-carbethoxyhistidine residues was determined by the increase in absorbance at 247 nm, using an extinction coefficient of 3200 M^{-1} min^{01} (Ovádi et al., 1967).

Restoration of Activity of the DEP-Inactivated Enzyme Using Hydroxylamine. After the inactivation of isocitrate lyase with varying concentrations of DEP and a reaction time of 3 min, excess DEP was destroyed by adding histidine (pH 6.8) to a concentration of 16 mM. Then the samples were treated with 0.2 M hydroxylamine at 4 °C for 24 h. Following the completion of this step the samples were gel filtered by sedimentation as described above and then assayed for isocitrate lyase activity.

Hydroxylamine Treatment. After 77 min of incubation of isocitrate lyase (25 μM in subunits) at 30 °C with DEP (0.5 mM) in PME buffer, pH 6.5, hydroxylamine was added to a final concentration of 0.2 M. The difference in absorbance of the sample treated with DEP and the control (enzyme alone) was determined at 240 nm.

Preparation of Labeled Peptides. Isocitrate lyase (21 μM in subunits) in PME buffer (pH 6.6) was incubated with 0.6 mM [^{3}H]DEP at 35 °C for 17 min and the reaction then quenched with 5.8 mM histidine (pH 6.6). In a parallel experiment, isocitrate lyase was incubated with 31 mM glyoxylate plus 31 mM succinate during and prior to the treatment with DEP, and quenching was conducted as just described. To the inactivated enzyme was added a large excess of *N*-ethylmaleimide (to 2.84 mM), and the mixtures were incubated for 10 min at 35 °C to alkylate the cysteine residues. The resulting alkylated enzyme preparations were denaturated by adding 5 mL of cold absolute ethanol to 1.7 mL of each sample and incubating for 5 min. Then, the samples were centrifuged for 22 min at 5000*g*, after which the resulting pellets were washed exhaustively with cold absolute ethanol. Each of the washed pellets was suspended in 2 mL of 25 mM MOPS (pH 7.0), trypsin was added (at a mass ratio of 1:50 with respect to isocitrate lyase), and the mixture was incubated for 6 h 20 min at 34 °C.

Separation of Labeled Peptides by HPLC. Separation of the peptides after trypsin digestion was accomplished by using HPLC with a C_8 reverse-phase column.

Table I: Substrate Protection of Isocitrate Lyase against Inactivation by DEP

sample treatment[a]	relative isocitrate lyase activity
control/no DEP treatment	(100)
DEP treatment only	24
12 mM D_s-isocitrate + DEP treatment	70
12 mM glyoxylate + DEP treatment	41
12 mM succinate + DEP treatment	29
6 mM glyoxylate + 6 mM succinate + DEP treatment	73
12 mM glyoxylate + 0.2 mM itaconate + DEP treatment	67

[a] Isocitrate lyase was preincubated with substrates individually in MME buffer at 37 °C, pH 6.8, for 5 min prior to and during incubation with 0.5 mM DEP for 3 min. Reactions were quenched with 16 mM His; the mixtures were gel filtered by sedimentation as described to remove excess reagents and products. Following this treatment the residual enzyme activity of each sample was determined.

Results

Saturation Kinetics for Inactivation by DEP. Isocitrate lyase was inactivated in a time-dependent manner when incubated with an excess of diethyl pyrocarbonate (DEP) dissolved in absolute ethanol. The linear relationship between $1/k_{obs}$ and 1/[DEP] (not shown) suggested that modification of the enzyme conforms to the kinetics scheme:

$$E + I \overset{K_i}{\leftrightarrow} EI$$

$$EI \xrightarrow{k_3} E'$$

where EI is a noncovalent enzyme–DEP complex and E′ is the inactivated enzyme. The inhibition constant (K_i) for DEP, which was the reciprocal of the *x*-intercept for the linear plot of $1/k_{obs}$ vs 1/[DEP], was found to be 0.7 mM; the reciprocal of the slope, the second-order rate constant (K_3/K_i), was 2.3 × 10^3 M^{-1} min^{-1}.

Substrate Protection of Isocitrate Lyase against Inactivation by DEP. The recovered isocitrate lyase activity was higher when inactivation had been carried out in the presence of substrates (Table I). The highest degree of protection was provided by D_s-isocitrate, glyoxylate plus succinate, or glyoxylate plus itaconate. Earlier research has suggested that the strong inhibitor itaconate is a succinate analogue (Rao & McFadden, 1965; Rittenhouse & McFadden, 1974; Tsukamoto et al., 1986). Some protection was afforded by glyoxylate alone but little or none by succinate alone. Concentrations of D_s-isocitrate, glyoxylate, or succinate employed (Table I) were well above values for K_m or K_i, which are 0.14, 0.28 and 0.45 mM, respectively, at pH 7.3 and 37 °C (Ko & McFadden, 1990b).

Effect of Time of Incubation of Isocitrate Lyase with DEP. The difference absorbance spectra of isocitrate lyase, after incubation with DEP for varying lengths of time, are shown in Figure 2; the absorbance maximum occurring at 247 nm

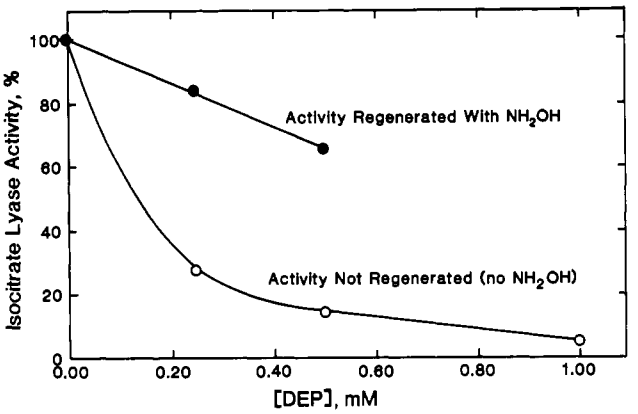

FIGURE 2: Restoration of activity to DEP-treated isocitrate lyase with NH_2OH. First, the enzyme was inactivated at 37 °C with varying concentrations of DEP (0, 0.25, 0.5, and 1.0 mM) for 3 min. Then, excess DEP was destroyed by adding His (pH 6.8) to a concentration of 16 mM, after which the samples were reacted with 0.2 M NH_2OH at 4 °C for 30 min. Following completion of this step the samples were gel filtered by sedimentation through columns of Sephadex G-50 that had previously been equilibrated with MME buffer (pH 7.3). Finally, assays for isocitrate lyase activity were made on the eluates.

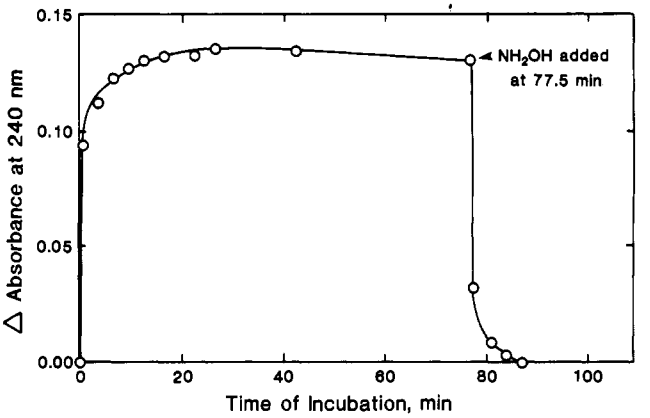

FIGURE 3: Effect of NH_2OH on DEP-inactivated isocitrate lyase. After 77 min of incubation of the enzyme (25 μM in subunits) with DEP (0.5 mM) in PME buffer at pH 6.5 and 30 °C, NH_2OH was added to a final concentration of 0.2 M. The difference in OD between the sample of enzyme treated with DEP and the control (isocitrate lyase alone) was determined at 240 nm. Hydroxylamine was added at the time indicated to both cuvettes.

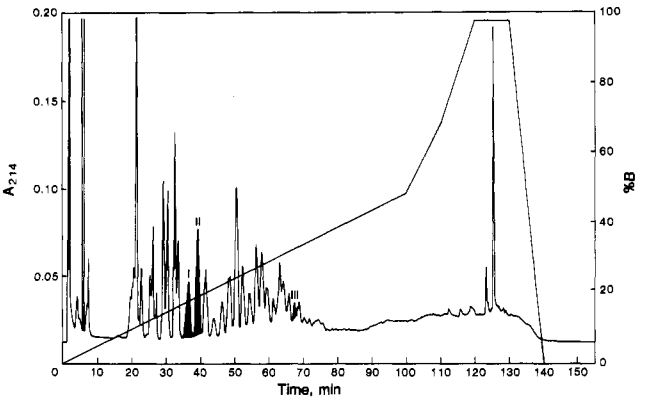

FIGURE 4: Isolation of carbethoxylated peptides by HPLC. Separation of peptides after the trypsin digestion was accomplished by using a C_8 reverse-phase column operating under the following conditions: flow rate, 1 mL/min; fraction size, 1 mL; solution A, deionized water; solution B, 70% acetonitrile; gradient, 0–50% B to 100 min, 50–70% B from 100 to 110 min, and 70–100% B from 110 to 120 min; absorbance at 214 nm was 0.20 at full scale.

suggests that mono-N-carbethoxylation of histidine residues occurs according to Avaeva and Krasnova (1975) and Miles (1977). Mono-*N*-carbethoxyhistidyl derivatives absorb maximally between 230 and 250 nm. Di-*N*-carbethoxyhistidyl derivatives have maxima between 220 and 240 nm (Miles, 1977). The absorbance difference reached a maximum after ca. 40 min of incubation (Figure 1) and corresponded to the modification of 2.0 histidine residues/subunit. Inexplicably, there was a slight increase in absorbance difference in the peak at approximately 282 nm.

Specificity of Modification and Effect of NH_2OH Treatment on DEP-Inactivated Isocitrate Lyase. Treatment of DEP-inactivated isocitrate lyase with NH_2OH restored a major portion of the original activity (Figure 2). The reaction of DEP with histidine residues of enzymes is known to give *N*-carbethoxyhistidyl derivatives that exhibit absorption maxima near 240 nm (Miles, 1977). Restoration of the activity of DEP-treated isocitrate lyase by hydroxylamine (Figure 2) implies the modification of either histidine or tyrosine residues and suggests that lysine is not involved (Melchior & Fahrney, 1970; Miles, 1977). That inactivation of isocitrate lyase arises from the modification of histidine and not tyrosine is indicated by the fact that there was no decrease in absorbance at 280 nm (Figure 1) as reported by Muhlrad et al. (1967); also the second-order rate constant of 2300 M^{-1} min^{-1} is similar to that for the reaction of imidazole with DEP in model systems or for the modification of histidine residues in most other proteins (Cousineau & Meighen, 1976). Moreover, much lower second-order rate constants have been observed for reactive lysine, cysteine, or tyrosine residues (Holbrook & Ingram, 1973; Wells, 1973).

In the present research, the inactivation of isocitrate lyase was carried out in the pH range of 6.5–6.8, where DEP is highly specific for histidine residues. Further supporting the specificity of histidine modification was the rapid decrease of absorbance at 240 nm after hydroxylamine treatment (Figure 3) whereas the decarbethoxylation of *O*-(ethoxyformyl)tyrosyl derivatives is known to occur at a much slower rate (Melchior & Fahrney, 1970). It has been reported that DEP reacts with *N*-acetylcysteine in carboxylate buffers with an increase in absorbance at 240 nm, which is also reversed by hydroxylamine (Garrison & Himes, 1975). In the present work, phosphate buffer was employed in spectral studies. Spectral modification of ribulose bisphosphate carboxylate/oxygenase (RuBisCO) or watermelon isocitrate lyase with DEP in phosphate buffer does not involve Cys residues (Saluja & McFadden, 1980; Jameel et al., 1985) suggesting that Cys is not modified in the present studies of carbethoxylation of isocitrate lyase by DEP.

Separation of Labeled Tryptic Peptides by HPLC. In numerous experiments three tryptic peptides were consistently labeled differentially by [^{3}H]DEP, i.e., contained 2–3 times more label after incubation of isocitrate lyase in the absence of glyoxylate and succinate than in the presence of these substrates. These peptides are designated I–III (Figure 4). In Figure 5, panels a and b, labeling in the presence of glyoxylate and succinate (31 mM each) is compared with that in their absence, respectively. Striking differential labeling was observed in peptides I–III. The sequence THAGIEQAISR was found in peaks I and II. Maximum radioactivity was seen in cycle 2 during sequencing of peak I, indicating that histidine had been labeled (Figure 6), and was seen for peak II also. Yields of radioactive derivatized His recovered in the second cleavage peak I were high (e.g., see the yield of 24% reported in Figure 6) considering the severity of sequencing conditions. Cyclization to form the phenylhydantoin derivative during sequencing requires a 20-min heat treatment of the derivatized peptide with 25% aqueous trifluoroacetic acid, yet DEP-modified His is only moderately stable. We

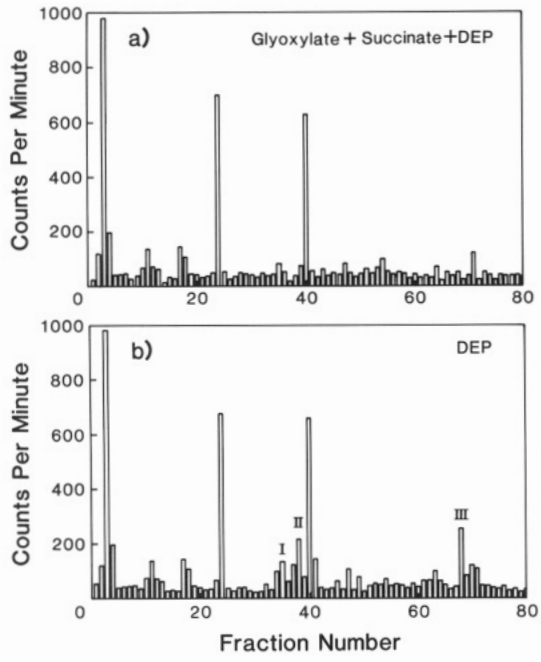

FIGURE 5: Distribution of radioactivity in the peptide fractions separated by HPLC. The cpm values for 50 μL of each 1.0-mL fraction are represented in the histograms and show the distribution of radioactivity from tritium decay for (a) inactivation with DEP conducted in the presence of substrates glyoxylate plus succinate (each at 31 mM) and (b) inactivation with DEP without substrates showing the increase in radioactivity in peaks I–III. Peaks I–III (panel b) arising from labeling in the absence of substrates were the most highly differentially labeled (cf. counterparts in panel a representing labeling in the presence of substrate). Apparent differential labeling of fraction 41 is illusory and probably represents a slight chromatographic mismatch between panels a and b and tailing of fraction 40. This differential labeling of fraction 41 was not consistently observed in other experiments and is therefore considered an artifact.

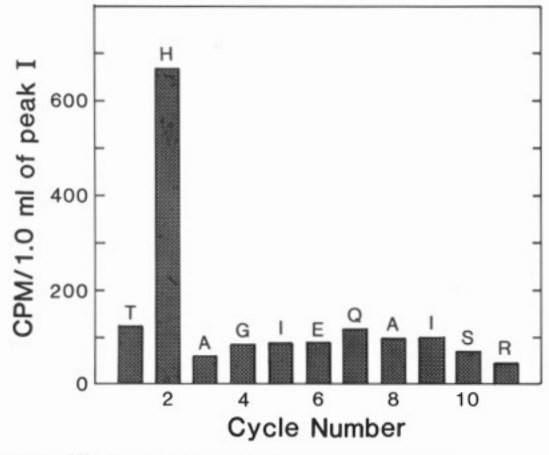

FIGURE 6: Histogram representing the residue identified and radioactivity collected with each cleavage of radiolabeled peptide peak I (based on 1.0 mL of peak I). The yield of derivatized histidine surviving sequencing procedures performed on peak I was 670/2800 (Figure 4), which converts to 24%. The sequence deduced (THAGIEQAISR) corresponds to residue stretch 265–275 of isocitrate lyase (Matsuoka & McFadden, 1988).

have no explanation for the occurrence of radioactive peptide in both peaks I and II (Figure 5) which contain identical sequences; perhaps, however, the histidine residue can be modified at either the N-1 or N-3 imidazole nitrogens to generate two different monocarbethoxylated derivatives. Unfortunately, the instability of these derivatives precluded further characterization.

The sequence FAQAIHAK (corresponding to residue stretch 301–308) predominated in peak III although a minor sequence INNTFR was also found. The latter corresponds to a tryptic peptide that would arise from residues 124–129, but this peptide could not have been labeled in isocitrate lyase that had been incubated with [^{3}H]DEP.

Discussion

The use of DEP to modify histidine residues is well-known [see, for example, Miles (1977), Schneider (1978), Saluja and McFadden (1980, 1982), and Lundblat and Noyes (1984)]. DEP, itself, is easily hydrolyzed. However, the carbethoxyhistidyl derivatives are somewhat stable at pH 7.0, with a maximal half-life up to 55 h. Although there are numerous reports of the use of DEP to assess the presence of essential enzymic histidine residues, there are few reports that identify which residue is modified by DEP (Hegyi et al., 1974; Igarashi et al., 1985) and in no case has labeling been verified by sequencing.

In studies of RuBisCO, [^{3}H]DEP has been used to label the spinach enzyme in the absence and presence of a strong competitive inhibitor, sedoheptulose 1,7-bisphosphate (SBP). Trypsinolysis followed by rapid fractionation of tryptic peptides and further rapid purification of labeled peptides has established that several are differentially labeled, i.e., more highly labeled when RuBisCO was modified in the absence of SBP. The major differentially labeled peptide has a composition corresponding to stretch 296–303 (Ala-Met-His-Ala-Val-Ile-Asp-Arg). On the basis of this approach, it was deduced that His298 is in the active site domain of spinach RuBisCO (Igarashi et al., 1985). The validity of this approach using [^{3}H]DEP has been confirmed by the recent finding based on crystallography that His298 is indeed in the active site and may participate in substrate binding (Knight et al., 1990).

In the present work, it is reported that DEP inactivates isocitrate lyase of *E. coli*. Two histidine residues per subunit are found to be carbethoxylated as determined by the increase in absorbance at 247 nm. The second-order rate constant (k_3/K_i) for the inactivation of isocitrate lyase by DEP of 2300 M^{-1} min^{-1} is very fast compared to that for other enzymes: 1 M^{-1} min^{-1} for chloroperoxidase (Blanke & Hager, 1990), 91–422 M^{-1} min^{-1} for D-xylose isomerase from several microorganisms (Vangrysperre et al., 1988), and 840 M^{-1} min^{-1} for uridine phosphorylase (Drabikowska & Wozniak, 1990). Saturation kinetics exhibited for the inactivation together with this high second-order rate constant suggest the presence of one or more very reactive histidine residues in or near the active site of isocitrate lyase. By keeping the working conditions at an optimum, histidine residues that are preferentially labeled in the absence of substrate have been identified. Optimal conditions involve the use of cold absolute ethanol to denature the labeled enzyme, enabling omission of prolonged dialysis to remove denaturants and labeled compounds, exclusion of trifluoroacetic acid in the HPLC separation of the tryptic peptides, rapid purification of radioactive peptides, and working, where possible, at a neutral pH.

The substrates, either isocitrate or glyoxylate plus succinate, protect the enzyme from inactivation. Glyoxylate, alone, provides some protection, but succinate provides little or none. From this, it appears that DEP enters both the glyoxylate and succinate substrate sites and reacts with histidine residues. The lack of protection by succinate may be due to the ordered kinetic mechanism wherein the binding of glyoxylate is a prerequisite for the binding of succinate (Ko & McFadden, 1990b).

Two histidine residues in *E. coli* isocitrate lyase appear to be preferentially labeled in the present work in the absence of substrates. Labeled DEP-modified His266 has been re-

Enzyme Source	Sequence										Reference
					266						
Ec	phe	phe	arg	thr	his	ala	gly	ile	glu	gln	Matsuoka & McFadden, 1988
Ct	tyr	tyr	arg	tyr	gln	gly	gly	thr	gln	cys	Atomi *et al.*, 1990
Bn	phe	tyr	arg	phe	gln	gly	ser	val	thr	ala	Comai *et al.*, 1989
Gh	phe	tyr	arg	phe	arg	gly	ser	val	met	ala	Turley *et al.*, 1990
Rc	phe	tyr	arg	phe	lys	gly	ser	val	met	ala	Beeching & Northcote, 1987
					390						
					306						
Ec	ala	gln	ala	ile	his	ala	lys	tyr	pro	gly	As above
Ct	ala	asp	gly	val	lys	ala	ala	val	pro	asp	As above
Bn	ala	glu	gly	val	lys	ser	lys	thr	pro	glu	As above
Gh	ala	ala	gly	val	lys	ser	met	his	pro	glu	As above
Rc	ala	glu	gly	val	lys	ser	met	his	pro	glu	As above
					430						

FIGURE 7: Flanking sequences around His266 and His306 of isocitrate lyase from *E. coli* (Ec), top line, compared with aligned sequences for the enzyme from yeast (*Candida tropicalus*, Ct), rapeseed (*Brassica napus*, Bn), cotton (*Gossypium hirsutum L.*, Gh), and castor bean (*Ricinus communis*, Rc). The alignments were achieved by using the GAP program of the University of Wisconsin Genetics Computer Group as described by Matsuoka and McFadden (1988).

covered after the second cleavage of radioactive tryptic peptides (I or II). The identification of His306 is based on detecting the appropriate sequence in the radioactive fraction. However, radioactivity was not observed in the sequencing cycle. This may have been due to the much smaller quantity of peptide analyzed, which was 20-fold lower than those containing His266, but also may have been due to loss of label from His306 during exposure to acid in the preceding sequencing cycles. In addition, His306 is close to the C-terminus of the tryptic peptide where lower recovery of the residue is expected because of sequentially lower yields.

Residue 266 is not conserved in aligned sequences of the enzyme from castor bean, rapeseed, cotton, or yeast, as shown in Figure 7. In plants, and presumably in yeasts, enzymes of the glyoxylate cycle are located within single membrane limited organelles termed glyoxysomes and are separated from isocitrate dehydrogenase which is localized in mitochondria. In contrast, in *E. coli* these two enzymes are cytosolic and compete for a common pool of isocitrate (Vanni et al., 1990); regulation of the flux through the tricarboxylic acid and glyoxylate cycles is achieved by reversible phosphorylation of the enzymes (Garnak & Reeves, 1979; Hoyt & Reeves, 1988). Isocitrate lyase is probably *activated by phosphorylation* of a His in the *E. coli* enzyme (Robertson et al., 1988), perhaps residue 266 (Matsuoka & McFadden, 1988), whereas isocitrate dehydrogenase is *inactivated by phosphorylation* of Ser113 (Malloy et al., 1984; Hurley et al., 1989). Given the compartmental separation of isocitrate lyase and isocitrate dehydrogenase in plants, it is probably significant that watermelon isocitrate lyase is not phosphorylated (Matsuoka & McFadden, 1988). If His266 is the site of phosphorylation of the *E. coli* enzyme, then it is not surprising that this residue is replaced by a Lys, Arg, or Gln in eukaryotic isocitrate lyases (Figure 7). Curiously, His266 appears to be in the active site of *E. coli* isocitrate lyase on the basis of the present work. Phosphorylation of this residue would introduce a negative charge that might decrease binding of the trianionic substrate isocitrate. Because activation apparently occurs with phosphorylation, one role of the active site Mg^{2+} (Vanni et al., 1990) may be to neutralize the phosphate moiety introduced during activation.

With respect to His306 of *E. coli* isocitrate lyase, which is also probably in the active site domain, the replacement by a cationic Lys residue in eukaryotic enzymes (Figure 7) is noteworthy. Perhaps this residue has a substrate-binding function in the *E. coli* enzyme and this function has been conserved in eukaryotic counterparts by the substitution of Lys.

To summarize, our present results suggest that one or more histidine residues in a functional domain of a protein may be differentially labeled with [^{3}H]DEP in the absence and presence of a ligand bound in this domain. Rapid fractionation of tryptic peptides and subsequent sequencing may result in unambiguous identification of modified histidine residue(s) even without further purification of the labeled peptides. The present results also suggest that [^{3}H]monocarbethoxylated histidine partially survives sequencing methodology and can be isolated and detected if it is toward the N-terminus of a tryptic peptide. These observations collectively open the way to the identification of histidine(s) in a functional protein domain.

Registry No. DEP, 1609-47-8; His, 71-00-1; isocitrate lyase, 9045-78-7.

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Tyrosine 70 Fine-Tunes the Catalytic Efficiency of Aspartate Aminotransferase†

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ABSTRACT: The aspartate aminotransferase mutant Y70F exhibits k_{cat} = 8% and k_{cat}/K_M = 2% of the wild type values for the transamination of aspartate and α-ketoglutarate. The affinity of the enzyme for the noncovalently bound inhibitor maleate is reduced 17-fold by the mutation, while only a 2.5-fold reduction is observed for α-methylaspartate, which forms a stable, covalent external aldimine. The high population of the quinonoid intermediate formed in the reaction of the wild type with β-hydroxyaspartate is more than 75% diminished by the mutation. The values of the Y70F C^{α}-H kinetic isotope effects for the aspartate reaction are larger than those of wild type (^{D}V = 2.4 vs 1.52; $^{D}(V/K)$ = 2.5 vs 1.7). Conversely, the Y70F value of $^{D}(V/K)$ for the glutamate reaction is decreased compared to wild type (1.75 vs 2.5). These results, combined with previous studies of Lys258 mutants, eliminate Tyr70 as an essential component of the catalytic apparatus, with the caveat that the functionality of the deleted hydroxyl group is possibly replaced by a water molecule.

Aspartate aminotransferase is a pyridoxal 5′-phosphate (PLP)[1] dependent enzyme that preferentially catalyzes the reversible interconversion of the dicarboxylic α-amino acids, L-aspartate and L-glutamate, and the corresponding α-keto acids, oxalacetate and α-ketoglutarate. It is the most extensively studied PLP-dependent enzyme involved in amino acid metabolism. Not only have numerous classical solution studies elucidated details of its mechanism but also X-ray crystallographic analyses have now provided three-dimensional atomic models around which this extensive body of data can be organized [for reviews see Jansonius and Vincent (1987) and Christen and Metzler (1985)]. The X-ray structure provided a basis for the formulation of a detailed mechanistic proposal (Kirsch et al., 1984), which is currently being tested by analyses of active site mutants generated by site-directed mutagenesis techniques (Cronin & Kirsch, 1988; Toney & Kirsch, 1989; Kuramitsu et al., 1987; Inoue et al., 1989).

The reaction pathway for aspartate aminotransferase includes several distinct intermediates (Scheme I). The chemically difficult step, and the one that differentiates transamination from other PLP-catalyzed reactions, is the 1,3 prototropic shift. It involves abstraction of a proton from C^{α} of the amino acid and its transfer to C4′ of the coenzyme. This step was originally proposed to be catalyzed by the active site residue Lys258 in concert with the hydroxyl group of Tyr70 (Kirsch et al., 1984). Considered most likely was the involvement of Tyr70 as a member of a charge-relay network between Lys258 and the coenzyme phosphate.

A direct probe of the function of Tyr70 in the AATase mechanism is provided by the site-directed mutant Y70F, in which Tyr70 is changed to Phe. Described herein are analyses of this mutant constructed with the *Escherichia coli* AATase. The bacterial enzyme facilitates molecular biological experiments. All active site residues in the prokaryotic and euka-

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[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); wild type, wild type *E. coli* AATase; Y70F, *E. coli* AATase in which Tyr70 has been changed to phenylalanine by site-directed mutagenesis; E-PLP and E-PMP, pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate forms of AATase, respectively; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; β-hydroxyaspartate, *erythro*-β-L-hydroxyaspartate; α-methylaspartate, α-methyl-D,L-aspartate; KIE, kinetic isotope effect.

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