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Isolation and Properties of N^{ϵ}-Hydroxylysine:Acetyl Coenzyme A N^{ϵ}-Transacetylase from *Escherichia coli* pABN11†

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ABSTRACT: The enzyme N^{ϵ}-hydroxylysine acetylase has been isolated from *Escherichia coli* 294 carrying recombinant plasmid ABN11. Activity of the enzyme was followed by measurement of the rate of appearance of 2-nitro-5-thiobenzoate, the product of cleavage of 5,5′-dithiobis(2-nitrobenzoate) by free coenzyme A released from its acetyl derivative. The enzyme bound firmly to Reactive Blue 2–Sepharose CL-6B and was eluated with 1.5 M KCl. The protein gave a single band, corresponding to a M_r of 33 000, on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In contrast, gel filtration of the native enzyme gave a M_r of 150 000–200 000. A sequence analysis of the DNA at the junction of the first and second genes in the aerobactin operon, considered in conjunction with the N-terminal amino acid sequence of the isolated protein, enabled the conclusion that the acetylase is specified by the second gene in the complex. The enzyme transfers the acetyl moiety from acetyl coenzyme A to a variety of hydroxylamines, with N^{ϵ}-hydroxylysine as the preferred substrate. In agreement with the results found by affinity chromatography, Coomassie Blue was observed to act as a potent inhibitor.

Siderophores, defined as virtually iron(III)-specific ligands, occur in nearly all aerobic and facultative anaerobic microbial species where they have been shown by both genetic and biochemical techniques to be involved in a high-affinity iron assimilation process (Neilands, 1984). Iron is a virulence factor for microorganisms pathogenic to man and animals (Weinberg, 1984), and the capacity to synthesize a specific siderophore, aerobactin, has been correlated with the invasive character displayed by certain clinical isolates of *Escherichia coli* (Warner et al., 1981). In plants, synthesis of siderophores by root-colonizing bacteria is thought to have a significant effect on growth (Schroth & Hancock, 1981). Iron, although usually a nutritionally essential and beneficial element, is at the same time responsible for the generation of oxidizing free radicals (Willson, 1984). For all of these reasons, siderophore-mediated assimilation of iron and the regulation of this process have assumed considerable interest in biology.

Substantial progress has been made in understanding the molecular genetics of a particular siderophore system commonly found in *E. coli* and related enteric bacteria. Thus, the aerobactin biosynthetic and transport complex encoded on plasmid ColV-K30 of hospital strains of *E. coli* has been cloned (Bindereif & Neilands, 1983), its main promoter has been identified (Bindereif & Neilands, 1985), and the general organization of the operon has been established (de Lorenzo et al., 1986). The genetic determinants of a regulatory protein, Fur (*Fe uptake regulation*), which controls at the the transcriptional level all siderophore-mediated iron uptake systems in *E. coli*, has been mapped (Bagg & Neilands, 1985), cloned (Hantke, 1984), and sequenced (Schaffer et al., 1985). Thus, the molecular mechanism of iron assimilation, and its regulation, may soon be known in some detail in bacteria, and it now remains to extend these studies to a eukaryotic species.

In contrast to bacteria, only limited techniques are currently available for gene cloning in fungi other than *Saccharomyces cerevisiae*, which, paradoxically, has not been shown to form a siderophore. One possibility is to isolate from a fungal species a pure biosynthetic or transport protein for a siderophore. The immunological properties of this protein or knowledge of its amino acid sequence can then be used to probe for its gene.

The enzyme performing the acetylation step in the synthesis of N^{δ}-acetyl-N^{δ}-hydroxyornithine, the iron(III)-binding amino acid common to ferrichrome and to many other hydroxamate-type siderophores of fungal origin, has been partially purified from the basidiomycete *Ustilago sphaerogena* (Ong & Emery, 1972). The acetylase synthesizing N^{ϵ}-acetyl-N^{ϵ}-hydroxylysine, the analogous next higher amino acid present

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in two residues per mole of aerobactin and mycobactin, has been obtained as a crude extract from *Aerobacter aerogenes* 62-I (*Klebsiella pneumoniae*) (Kusters & Diekmann, 1984), the original source of aerobactin (Gibson & Magrath, 1969). In the case of *E. coli* pColV-K30, we found by means of a ^{14}C label assay similar to that used by Kusters and Diekmann (1984) that the enzyme is derived from gene *iuc*B of the cloned aerobactin operon and is a polypeptide with M_r[1] of 33 kDa (de Lorenzo et al., 1986).

We now describe the isolation from *E. coli* 294 (pABN11) of N^{ϵ}-hydroxylysine:acetyl coenzyme A N^{ϵ}-transacetylase (acetylase) in the pure state and record some of the properties of the enzyme. The reaction catalyzed is

$$HO_2C-CH(NH_2)-CH_2CH_2CH_2CH_2-NH(OH) \xrightarrow{AcCoA} HO_2C-CH(NH_2)-CH_2CH_2CH_2CH_2-N(OH)-COCH_3$$

This is the first reported isolation of a homogeneous protein catalyzing any step in siderophore synthesis. We anticipate that this may facilitate isolation of the corresponding acetylase from fungal sources and, in turn, the iron-regulated genes. Experiments are in progress with the basidiomycetous yeast *Rhodotorula pilimanae*, which forms the siderophore rhodotorulic acid, to achieve this goal (Liu & Neilands, 1984).

Experimental Procedures

Materials. The following materials were obtained from the commercial sources indicated. Coenzyme A (CoA) (Na salt), *n*-propionyl-CoA (Li salt), isobutyryl-CoA (Li salt), human hemoglobin (grade IV), cytochrome *c* (type III, horse heart), soybean β-amylase, BSA (fraction V), Reactive Blue 2–Sepharose CL-6B, Sepharose CL-6B, and SDS-70L molecular weight protein standards were from Sigma Chemical Co. Ferritin (equine spleen) was from Calbiochem. Sepharose 6B was from Pharmacia Fine Chemicals. Protein assay dye reagent and gel electrophoresis reagents were from Bio-Rad. PM10 ultrafiltration membranes were from Amicon Corp. All other materials were of the highest quality available.

Bacterial Strain and DNA. E. coli 294 carrying pABN11 (de Lorenzo et al., 1986), a plasmid with a 0.3-kb deletion at the two closely spaced *Bam*HI sites in the midregion of the 6.8-kb insert of pABN5 (Bindereif & Neilands, 1983), was used as a source of acetylase. A 1.0-kb *Pst*I fragment of pABN15 (de Lorenzo et al., 1986), the latter a recombinant plasmid carrying the first gene and a small segment of the second gene of the operon, was ligated into the *Pst*I site in M13-mp9 (Messing & Vieira, 1982). This construction was partially sequenced by the chain-terminator method (Sanger et al., 1977).

Assay Procedures. Protein concentrations were determined by the method of Bradford (1976), with BSA as standard. Stock CoA solution was standardized by adding 0.1 mL of a solution containing 0.39 mg/mL DTNB in 1 M Tris-HCl, pH 8.1, to the sample, and the volume was adjusted to 1.0 mL with water. The concentration of 2-nitro-5-thiobenzoate was determined from the ϵ at 412 nm of 13 600 M^{-1} cm^{-1} (Srere, 1969). For routine assays, the method of Srere (1969) was used to monitor the rate of appearance of CoASH, as catalyzed by N^{ϵ}-hydroxylysine acetylase in the presence of the DL-N^{ω}-hydroxy derivatives of lysine or ornithine at 3 mM concentration. At pH values below 8.1, CPDS was substituted for DTNB. The following buffers were made up as stock 1 M solutions: pH 6.0, histidine hydrochloride; pH 6.5, pH 7.0, and pH 7.5, sodium phosphate; pH 8.1 and pH 8.5, Tris-HCl; pH 9.0, sodium borate. At pH 7.0, the ϵ for CPDS at 344 nm with stock CoA as standard was estimated to be 10 000 M^{-1} cm^{-1}. A unit of activity was defined as the amount of enzyme giving 1 μmol of CoASH/min at 25 °C and pH 7.0.

Substrates and Inhibitors. Synthetic DL-N^{ϵ}-hydroxylysine and DL-N^{δ}-hydroxyornithine were prepared as described (Rogers & Neilands, 1963). The L forms of the amino acids and of 1-amino-5-(*N*-hydroxyamino)pentane were obtained by 6 N HCl hydrolysis of aerobactin (Warner et al., 1981), rhodotorulic acid (Atkin & Neilands, 1968), and deferriferrioxamine B, respectively. The mesylate salt of the last named siderophore, Desferal, was purchased from Ciba-Geigy Chemical Co. *N*-Methylhydroxylamine hydrochloride was prepared by reduction of nitromethane (Kjellin, 1893), and hydroxylamine hydrochloride was purchased from Eastman Chemical Co. The concentration of solutions of hydroxylamines was estimated by a modified Csaky test (Gillman et al., 1981), with omission of acid hydrolysis, and from the step height of the pK_a at pH ~5 as measured by potentiometric titration. Coomassie Brilliant Blue R (Sigma Chemical Co.) was used without further purification for the inhibitor studies. A 50% purity, as specified by the supplier, was assumed.

Isolation Procedure. E. coli 294 (pABN11) was cultivated in Tris–succinate medium (Warner et al., 1981) containing 0.3 M Tris, 4 g of sodium succinate, and 100 mL of Luria broth per liter. After supplementation with 30 mg of ampicillin and 10 mg of thiamin chloride–HCl, the sterilized medium was inoculated with 5 mL of an overnight culture grown up in Luria broth containing ampicillin. After 18 h at 37 °C on a rotary shaker, the cells were separated by centrifugation, washed with 50 mM potassium phosphate, pH 7, and resuspended in 10 mL of the same buffer. The cells were disrupted by sonications, and the debris was removed by centrifugation at 27000*g*. The supernatant extract was then centrifuged at 180000*g* for 1 h in the T865 rotor in order to remove fine particles. The crude extract was dialyzed overnight against 0.1 M potassium phosphate, 0.15 M NaCl, and 0.02% NaN_3, pH 7.0, and applied to a 5-mL column of Reactive Blue 2–Sepharose CL-6B that had been swollen and equilibrated in the same buffer. Chromatography was performed at room temperature with collection of 5-mL fractions. The flow rate was 5 mL/h during application and 10 mL/h during elution. The column was washed with 50 mL of dialysis buffer. Elution was achieved with a stepwise KCl gradient. Fractions containing N^{ϵ}-hydroxylysine acetylase activity were pooled and dialyzed exhaustively against 50 mM potassium phosphate, pH 7.0. The pooled, dialyzed fractions (~170 mL) were concentrated at 4 °C by ultrafiltration on an Amicon apparatus fitted with a PM10 membrane and operated at 30 psi.

N-Terminal Amino Acid Sequence. Sequencing was performed with a Beckman 890C sequencer. A Beckman HPLC analyzer was used for analysis of the residues. Six cycles were carried out.

Molecular Weight Determinations. The method of Francis (1984) was used for SDS–PAGE analysis, with a 6.5% stacking gel, a 10% running gel, and Coomassie Blue stain. Analytical gel filtration was carried out with Sepharose 6B in 0.1 M potassium phosphate, 0.15 M KCl, and 0.02% NaN_3, pH 7.0, in a 1.5 × 102 cm column. Chromatography was performed at room temperature with collection of 1.5-mL fractions. Ferritin (443 000), β-amylase (200 000), hemoglobin

[1] Abbreviations: CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoate); CPDS, carboxypyridine disulfide or 6,6′-dithiodinicotinic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kb, kilobase, kDa, kilodalton; M_r, relative molecular weight in SDS–PAGE; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Purification of N^{ϵ}-Hydroxylysine Acetylase[a]

fraction	activity (units)	protein (mg)	sp act. (units/mg)	purification (x-fold)	yield (%)
dialyzed crude extract	56	207	0.27	1	100
1.5 M KCl eluate from Blue Sepharose	49	1.85	26	97	87
concentrated protein	35	1.37	26	97	72

[a] Data are for 1 L of culture.

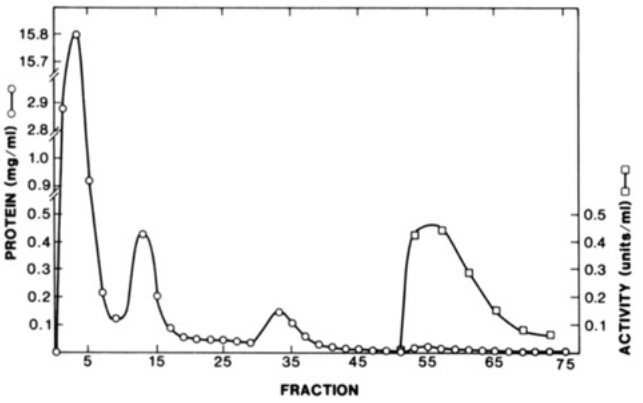

FIGURE 1: Elution profile for total protein and N^{ϵ}-hydroxylysine acetylase activity from a Reactive Blue–Sepharose CL-6B column. Step elutions of 0.5, 1.0, and 1.5 M potassium chloride in 0.1 M phosphate buffer, pH 7.0, were applied between fractions 11–30, 31–51, and 52–69, respectively.

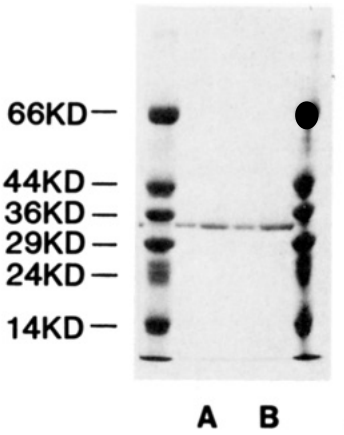

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of isolated N^{ϵ}-hydroxylysine acetylase, stained with Coomassie Blue. Molecular weight markers are on both sides of the figure. (A) Preparation 1-20-85, 2 and 5 μg; (B) Preparation 2-8-85, 2 and 5 μg.

CTGCAGGCTCAGGAGGTCTGAATGTCTGGGGCAAACATTGTT.....

Predicted amino acid sequence:	Met	Ser	Gly	Ala	Asn	Ile	Val
Amino acid residue found:	-	Ser	Gly	Ala	Asn	Ile	Val
Nanomoles/residue:	-	0.6	1.0	1.6	1.3	1.4	1.4

FIGURE 3: Correlation of base sequence of gene *iuc*B of the aerobactin operon with the N-terminal amino acid sequence of isolated N^{ϵ}-hydroxylysine acetylase. The upstream *Pst*I and putative Shine–Dalgarno ribosome binding sites are marked by single and double lines, respectively. The first base of the *Pst*I site shown occurs 204 bp upstream from the first base of the *Ava*I site of pABN5 (Bindereif & Neilands, 1983).

(63 000), cytochrome *c* (13 400), and Blue Dextran were used to calibrate the column.

Kinetics. Initial velocity data for determination of kinetic parameters and substrate specificity were fitted to the equation

$$v_i = V_{max}/[K_M(\text{acetyl-CoA})/[\text{acetyl-CoA}] + K_M(\text{substrate})/[\text{substrate}] + 1]$$

by using a SAS nonlinear least-squares iterative phase program provided by Dr. Jack Kirsch. Initial velocity data for studies of inhibition of Coomassie Blue were graphically analyzed as in Cornish-Bowden (1976), with linear regression to evaluate the slopes and intercepts of the lines.

RESULTS

N^{ϵ}-Hydroxylysine Acetylase. Figure 1 shows the elution profile obtained by affinity chromatography over Reactive Blue 2–Sepharose. All of the enzyme activity was recovered in the 1.5 M KCl elution fractions. The activity failed to bind to Sepharose CL-6B and was not eluted from the Reactive Blue 2–Sepharose at 1 mM acetyl-CoA, a concentration sufficient to saturate the enzyme.

Table I summarizes the results of the purification and concentration steps; an overall yield of 67% was obtained. The concentrated protein was pure by the criteria of a single band on SDS–PAGE analysis (Figure 2) and a single peak on gel filtration (Figure 4).

Sequence Analysis. Figure 3 summarizes data for sequence analysis of the DNA at the upstream end of the second gene (*iuc*B) in the aerobactin operon and for the amino acid analysis six residues into the protein from the *N*-terminal end. The sequences immediately upstream from the ATG start codon contain a strong ribosome binding site, AGGAGGT, complementary to a sequence near the 3′ end of 16*S* RNA, as originally proposed by Shine and Dalgarno (1975). The sequence between the *iuc*B ribosome binding site and the presumed *iuc*A stop codon spans only 4 bp, and no region resembling the *E. coli* promoter concensus sequence (Hawley & McClure, 1983) was found. Most likely, transcription of the acetylase gene (*iuc*B) is driven by the main, strong, iron-regulated promoter preceding the first gene (*iuc*A) in the aerobactin operon (Bindereif & Neilands, 1985).

Physical Properties of the Enzyme. SDS–PAGE analysis indicates a subunit size of about 33 kDa (Figure 2). Analytical gel filtration of the enzyme, however, reveals that the native enzyme may exist as a M_r 150 000–200 000 multimer containing several units of the protomer (Figure 4). The enzyme appears to be quite stable and can be kept at room temperature for several days and at 4 °C for at least several months without loss of activity.

Kinetics Properties of the Enzyme. The standard conditions for examination of kinetic parameters were 25 °C and pH 7. Measurement of initial velocity in buffers ranging from pH 6 to pH 9 indicated an optimum close to pH 7.0. With temperature as variable, the velocity rose to a maximum at 40 °C and declined sharply at higher temperatures (data not shown).

The kinetics parameters of the enzyme, with acetyl-CoA and a number of different hydroxylamines, are recorded in Table II. Although N^{ϵ}-hydroxylysine is the best substrate by the criterion of having the highest k_{cat}/k_m value, the enzyme is able to acetylate a number of hydroxylamines. L-Lysine and isobutyryl-CoA were found to be neither inhibitors nor substrates, while propionyl-CoA was not more than 5% as active as acetyl-CoA. In the presence of excess acetyl-CoA and enzyme, DL-N^{ϵ}-hydroxylysine was fully acetylated. A possible kinetic discrimination against the D isomer was not investigated.

Inhibition by Coomassie Blue. Previous workers have shown that Reactive Blue 2–Sepharose, as well as related gels, bound

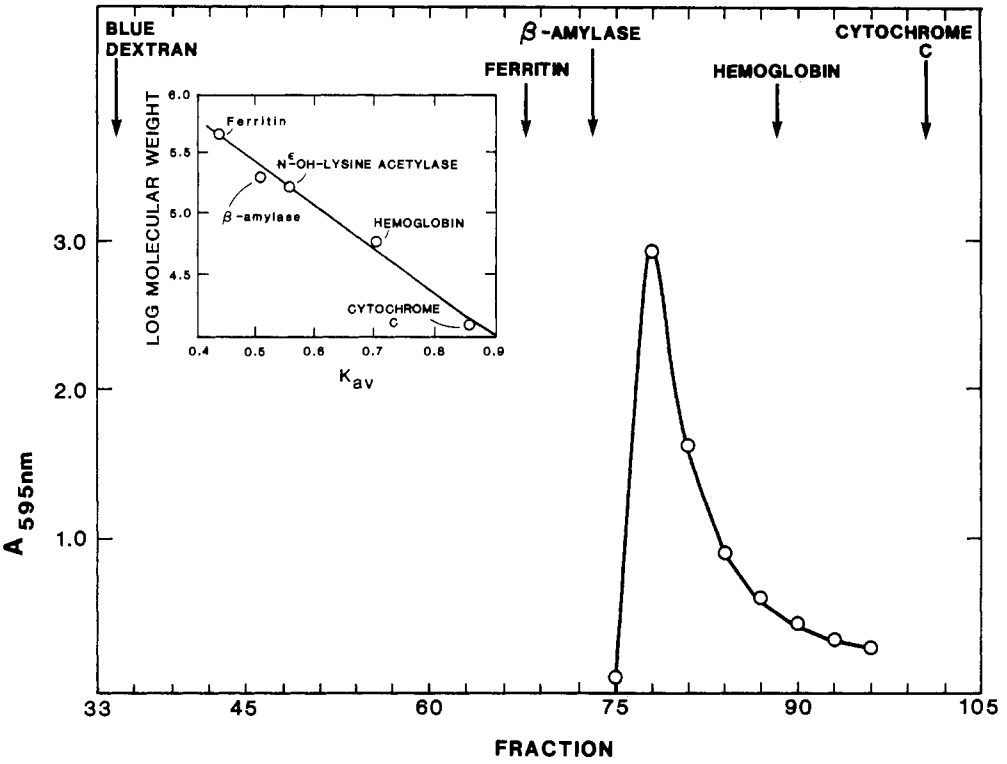

FIGURE 4: Gel filtration chromatography of isolated N^{ϵ}-hydroxylysine acetylase on Sepharose 6B. The A_{595} values were obtained by use of 0.2-mL samples in the Bradford (1976) microassay for protein.

Table II: Kinetic Parameters of N^{ϵ}-Hydroxylysine Acetylase[a]

substrate	K_M(acetyl-CoA) (mM)	K_M(RNOH) (mM)	k_{cat} ($\times 10^{-3}$ min^{-1})[b]	k_{cat}/K_M ($\times 10^{-3}$ min^{-1} mM^{-1})
L-N^{ϵ}-hydroxylysine	0.043 ± 0.005	0.055 ± 0.005	2.86 ± 0.12	52.0 ± 5.1
L-N^{δ}-hydroxyornithine	0.079 ± 0.013	0.317 ± 0.052	6.71 ± 0.66	21.2 ± 4.0
1-amino-5-(hydroxyamino)pentane	0.099 ± 0.017	0.330 ± 0.049	4.78 ± 0.42	14.5 ± 2.5
N-methylhydroxylamine	0.101 ± 0.029	0.396 ± 0.068	2.31 ± 0.31	5.8 ± 1.3
hydroxylamine	1.81 ± 0.830	329.4 ± 152.9	106.3 ± 47.1	0.32 ± 0.21

[a] Values are reported ± 1 SD. [b] k_{cat} values were calculated by assuming a 33-kDa molecular mass and are for purposes of comparison only.

enzymes requiring nucleotide-containing coenzymes; the bound enzymes could be eluted from the gel with the coenzyme (Ashton, 1978). The enzymes have been generally thought to bind to the dye-substituted matrix at the "dinucleotide" fold, a conserved structural domain responsible for coenzyme binding. Since the N^{ϵ}-hydroxylysine acetylase activity cannot be eluted from Reactive Blue 2–Sepharose with acetyl-CoA, the basis for binding is in this case unclear. Ashton (1978) found that Reactive Blue 2 and many other sulfonated aromatic dyes, including Coomassie Blue, acted as competitive inhibitors of the substrate for a cyclic nucleotide phosphodiesterase, an enzyme that was bound by Reactive Blue 2–Sephacryl S-200.

To investigate the basis for the binding of N^{ϵ}-hydroxylysine acetylase to Reactive Blue 2–Sepharose, the K_M(acetyl-CoA) was measured as a function of Coomassie Blue concentration. The dye was found to act as a mixed inhibitor of the acetylase with respect to acetyl-CoA, with $K_I = 0.71$ μM and $K_I' = 4.1$ μM. This contrasts with the results of Ashton (1978) with phosphodiesterase, where the inhibitor was purely competitive. Little effect was seen on $K_M(N^{\delta}$-hydroxyornithine) at saturating acetyl-CoA concentrations in the presence of the dye (data not shown.) Since K_I is $< K_I'$, Coomassie binds to the enzyme alone better than to the enzyme–acetyl-CoA complex, suggesting that acetyl-CoA and Coomassie Blue share binding sites on the enzyme. This may indicate that the N^{ϵ}-hydroxylysine acetylase binds to Reactive Blue 2–Sepharose at the acetyl-CoA binding site.

Discussion

Iron is required for energy metabolism and for other essential cellular functions, such as DNA synthesis, but for reasons already stated, it may also be a noxious element. We cloned the aerobactin complex of plasmid ColV-K30 in the expectation that the relative simplicity of the siderophore system would facilitiate investigation of its mode of regulation by iron (Bindereif & Neilands, 1983). We also hoped that the cloned sequences containing genes for amine oxygenation and hydroxylamine acetylation could be used to probe for the corresponding genes in fungi. However, we have thus far been unable to detect hybridization of the labeled insert of pABN5 with a restriction digest of DNA from *R. pilimanae*, the fungus synthesizing rhodotoluric acid. At this point, we turned to isolation of a pure protein from the aerobactin pathway, and the enzyme performing acetylation of N^{ϵ}-hydroxylysine was selected for the following reasons. Ong and Emery (1972) had found an enzyme-catalyzed, CoA-dependent acetylation of N^{ϵ}-hydroxylysine in the ferrichrome synthesis pathway in *U. sphaerogena*, and Kusters and Diekmann (1984) had shown that acetylase activity could be extracted from *A. aerogenes*. Among our collection of recombinant plasmids, pABN11 seemed the best choice as a source of the enzyme since minicell preparations from bacteria harboring this plasmid gave in SDS–PAGE analysis a strong band that by deletion could be

correlated with acetylase activity (de Lorenzo et al., 1986).

What was unanticipated was the exceptionally strong affinity of N^{ϵ}-hydroxylysine acetylase for Reactive Blue 2–Sepharose CL-6B. This factor, in combination with the magnified level of expression from the high copy number pABN11 and the strength of the aerobactin promoter (Bindereif & Neilands, 1985), facilitated isolation of the pure enzyme in a single fractionation step. Regulation of the aerobactin gene complex by iron is attenuated in these recombinant plasmids (Bindereif & Neilands, 1983), a fact that led to overproduction of the *iuc*B gene product in media not particuarly low in iron. The agreement of the DNA and protein sequences shown in Figure 3 proves that the acetylase is coded by *iuc*B, the second gene in the operon.

The DTNB method appears to be especially well suited for assay of N^{ω}-hydroxyacetylase activity, indicating lack of inhibition of the enzyme by the reagent. Recently, we have found this assay to be applicable to the N^{δ}-hydroxyornithine acetylase extracted from *R. pilimanae*, although in this case the enzyme is by comparison less tightly bound to the gel. Regarding specificity, our data are compatible with the report of Kusters & Diekmann (1984), who found that the acetylase of *A. aerogenes* acts on the N^{ω}-hydroxy forms of both lysine and ornithine.

As anticipated from its behavior or Reactive Blue–Sepharose, commercial Coomassie Blue proved to be a potent inhibitor. Selective inhibitors of aerobactin biosynthesis could be of significant value in combating infections caused by virulent strains of *E. coli*.

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Registry No. $HONH(CH_2)_5NH_2$, 929-24-8; HONHMe, 593-77-1; NH_2OH, 7803-49-8; AcCoA, 72-89-9; N^{ϵ}-hydroxylysine acetylase, 101077-53-6; L-N^{ϵ}-hydroxylysine, 19460-99-2; L-N^{δ}-hydroxyornithine, 35187-58-7; Coomassie blue, 78642-64-5.

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