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Lysine: N^6-Hydroxylase: Stability and Interaction with Ligands

Scott Dick,[1] Laura Marrone,[1] Henry Duewel,[2] Michael Beecroft,[1] Jennifer McCourt,[1] and Thammaiah Viswanatha[1,3]

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Recombinant lysine:N^6-hydroxylase, *r*IucD, which is isolated as an apoenzyme, requires FAD and NADPH for its catalytic function. *r*IucD preparations have been found to undergo time-dependent loss in monooxygenase function due to aggregation from the initial tetrameric state to a polytetrameric form(s), a process which is reversible by treatment with thiols. Ligand-induced conformational changes in *r*IucD were assessed by monitoring its CD spectra, DSC profile, and susceptibility to both endo- as well as exopeptidases. The first two methods indicated the absence of any significant conformational change in *r*IucD, while the last approach revealed that FAD, and its analog ADP, can protect the protein from the deleterious action of proteases. NADPH was partially effective and L-lysine was ineffective in this regard. Deletion of the C-terminal segment, either by treatment with carboxypeptidase Y or by mutagenesis of *iucD,* results in the loss of *r*IucD's monooxygenase activity. These findings demonstrate the crucial role of the C-terminal segment in maintaining *r*IucD in its native conformation.

KEY WORDS: Lysine monooxygenase; flavoprotein; mutagenesis; protease probes; ligand protection; C-terminus; protein stability.

1. INTRODUCTION

Lysine:N^6-hydroxylase, IucD,[4] catalyzes the conversion of L-lysine to its N^6-hydroxy derivative, the initial event in the biosynthesis of the siderophore aerobactin (Gibson and Magrath, 1969; Gross *et al.*, 1985; de Lorenzo *et al.*, 1987; Viswanatha *et al.*, 1987; Goh *et al.*, 1989; Thariath *et al.*, 1993*a*). Recently, gene fusion strategies based on the introduction of a hydrophilic peptide segment at the N-terminus of the *iucD* gene product have led to the production of recombinant forms of the protein (Thariath *et al.*, 1993*a,b,* 1994; Stehr *et al.*, 1999). The apo-*r*IucD preparation requires FAD and NADPH for its catalytic function (Thariath *et al.*, 1993*a,b,* 1994) and is stringently specific with L-lysine serving as its preferred hydroxylatable substrate (Marrone *et al.*, 1996). A study of

[1] Department of Chemistry, University of Waterloo, Waterloo N2L3G1, Canada.

[2] College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065.

[3] To whom correspondence should be addressed; E-mail: tviswan @sciborg.uwaterloo.ca.

[4] Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; CD, circular dichroism; CPDY, carboxypeptidase Y; DFP, diisopropylfluorophosphate; dNTP, deoxynucleotide triphosphate; DSC, differential scanning calorimetry; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DTT, DL-dithiothreitol; EDTA, ethylenediamine tetraacetic acid; ESMS, electrospray mass spectrometry; ε_M, molar extinction coefficient; FAD, flavin adeninc dinucleotide (oxidized form); G-6-P, glucose-6-phosphate; GDP, guanosine diphosphate; *iucD*, gene encoding for lysine:N^6-hydroxylase; IucD, lysine:N^6-hydroxylase, *iucD* gene product; mutein, protein with amino acid replacements or deletions; $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); pAT5, plasmid bearing *riucD;* PIPES, piperazine-N,N^6-bis(2-ethanesulfonic acid); PMSF, phenylmethane sulfonyl fluoride; *r*IucD, recombinant apo form of lysine:N^6-hydroxylase; Leu409ter *r*IucD, *r*IucD Δ17; Leu412ter *r*IucD; *r*IucD Δ14; Pro415ter *r*IucD, *r*IucD Δ11; Leu418ter *r*IucD, *r*IucD Δ8; SBTI, soybean trypsin inhibitior; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLCK, N-tosyl-L-lysyl chloromethyl ketone; and TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

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the reactivity and the function of the cysteine residues in *r*IucD revealed that three of the five residues present are accessible to reaction with DTNB in the native conformation of the protein. However, under identical experimental conditions, only two, Cys51 and Cys158, are susceptible to alkylation by ICH_2COO^-. Such chemical modification of *r*IucD is accompanied by a loss of its lysine:N^6-hydroxylase activity, while its ability to promote NADPH oxidation remains unaffected. Interestingly, replacement of the above-mentioned alkylatable cysteine residues with alanine has no adverse effect on the protein's catalytic functions (Marrone and Viswanatha, 1997). Thus, it would appear that the preferential disappearance of *r*IucD's monooxygenase function upon chemical modification of its cysteine residues may be a reflection of imposition of constraint(s) on its ability to undergo substrate-induced conformational transition(s) and/or cofactor-induced conformational changes which are inherent features of enzyme-catalyzed processes. In the case of monooxygenases, this phenomenon has been elegantly illustrated in studies with *p*-hydroxybenzoate hydroxylase (Gatti *et al.,* 1994) and phenol hydroxylase (Enroth *et al.,* 1998), which have been shown to alter their flavin cofactor environment in response to the presence of their substrates. Current investigations concern an assessment of cofactor- and/or substrate-induced conformational changes in *r*IucD by a study of its susceptibility to degradation by proteases.

2. MATERIALS AND METHODS

2.1. Materials

ADP, GDP, FAD, $NADP^+$, NADPH, G-6-P, G-6-P dehydrogenase, DTT, mercaptoethanol, TPCK-trypsin, TLCK chymotrypsin, and azocasein were purchased from Sigma (St. Louis, MO); media reagents for *E. coli* cultures were DIFCO products. CPDY was obtained from Calbiotech (Copenhagen). Furylacryloyl-Phe-Ala was obtained from Bachem Biosciences (Philadelphia, PA). Agarose, chromatography media, alkaline phosphatase detection kit, and goat anti-rabbit IgG were from Bio-Rad (Richmond, CA). Custom primary rabbit anti *r*IucD antibody was from Alpha Diagnostic International (San Antonio, TX). *Pwo* DNA polymerase was obtained from Boehringer Mannheim (Mannheim, Germany). dNTPs were purchased from Pharmacia (Uppsala, Sweden). The details regarding the construction of pAT5 harboring *iucD* encoding for *r*IucD have been given (Thariath *et al.,* 1993*a*).

2.2. Production of Truncated Derivatives of *r*IucD

Incorporation of a termination triplet, TGA or TAA, at specific locations in *iucD* by site-directed mutagenesis provided the approach for the generation of truncated *r*IucD variants. Such site-directed mutagenesis of *iucD* was achieved with the aid of a QuickChange* mutagenesis kit obtained from Stratagene cloning system (La Jolla, CA) employing protocols recommended by the supplier (Instruction Manual, Catalog #200518; Revision #25001). The resourcefulness of this procedure in achieving replacement of cysteine residues of *r*IucD has recently been reported from our laboratory (Marrone and Viswanatha, 1997). The nucleotide sequences of mutagenic, complementary primers employed for site-directed mutagenesis of *iucD* are given in Table I.

The protocols observed in the introduction of the desired mutations in *iucD* by PCR are similar to those reported previously from this laboratory (Marrone and Viswanatha, 1997). The PCR product, following treatment with *Dpn*I to degrade the parent plasmid, was used to transform *E. coli* (DH5α) and the transformants were selected on the basis of ampicillin resistance. The preparation and purification of the plasmid from these transformed cells was achieved by the alkali extraction method (Birnboim and Doly, 1979) using protocols outlined in Qiagen Plasmid Handbook (Chatsworth, CA). The successful incorporation of the mutation was assessed by the determination of the nucleotide sequence of *iucD* in the plasmid preparation with the aid of the following primers:

1. M13/pUC sequencing primer (≈20) (17-mer) 5′-d(GTAAAACGACGGCCAGT)-3′
2. 5′-d(CCGCAATATCTGCCTTGG)-3′ (426–443) (18-mer)
3. 5′-d (GGAAAGTAGTGGTCCTGG)-3′ (900–917) (18-mer)
4. M13/pUC reverse sequencing primer (≈24) (16-mer), 5′-d(AACAGCTATGACCATG)-3′

The nucleotide sequence determination was performed by Mobix Central Core Facility, McMaster University, Hamilton, Ontario, Canada, employing the dideoxynucleotide method (Sanger *et al.,* 1977).

2.3. Synthesis and Purification of the Decapeptide (C-Peptide)

The decapeptide Pro-Ala-Leu-Ile-Gln-Trp-Arg-Ser-Gly-(D)-Thr, with the amino acid sequence corresponding to that of the C-terminal segment (residues 416–425) of *r*IucD except for a change in the configuration of the terminal threonine residue, was synthe-

Table I. Nucleotide Sequence of Complementary Mutagenic Primers Used for Mutagenesis of *iucD*[a]

Plasmid		Complementary primers	Mutation in *r*IucD	Protein
pAT5	(1)	5′d(GTAATGGGACGTGATTAATTCGATCTCAGTATG)3′	L409-ter	*r*IucD Δ17
	(2)	5′d(CATACTGAGATCGAATTAATCACGTCCCATTAC)3′		
pAT5	(1)	5′d(CGTGATTTATTCGATTAGAGTATGCCGCCCGCC)3′	L412-ter	*r*IucD Δ14
	(2)	5′d(GGCGGGCGGCATACTCTAATCGAATAAATCACG)3′		
pAT5	(1)	5′d(TTCGATCTCAGTATGTAGCCCGCCCTGATTCAG)3′	P415-ter	*r*IucD Δ11
	(2)	5′d(CTGAATCAGGGCGGGCTACATACTGAGATCGAA)3′		
pAT5	(1)	5′d(CTCAGTATGCCGCCCGCCTAGATTCAGTGCCGCAGC)3′	L418-ter	*r*IucD Δ8
	(2)	5′d(GCTGCGCCACTGAATCTAGGCGGGCGGCATACTGAG)3′		

[a] The plasmid pAT5 (Thariath *et al.*, 1993a) was used to effect deletion at the desired location. The incorporation of the termination triplet at the desired location was achieved by the use of complimentary primers (1) and (2) containing the deletion codon (underlined).

sized by Dr. B. A. K. Chibber (Central Bio-core Facilities, University of Notre Dame, Notre Dame, IN). The material was purified by HPLC on a 0.8 × 10 cm Bondapak C_{18} RCM column employing a solvent system comprising water (0.1% TFA) and CH_3CN (0.1% TFA), with the latter being brought to 25% and 90% after 40 and 50 min, respectively, of the initiation of the procedure (flow rate, 1 ml/min; temperature, 25°C). The material was characterized by ESMS analysis. Calculated: 1128.3; found, 1127.4.

2.4. Isolation and Purification of *r*IucD and Muteins

The isolation and purification of *r*IucD and its muteins was achieved according to the procedures documented earlier (Thariath *et al.*, 1993*a;* Marrone and Viswanatha, 1997).

2.5. Determination of Protein and DNA Concentration

The concentration of *r*IucD was determined spectrophotometrically using an ϵ_M value of 6.2×10^4 M^{-1} cm^{-1} (Marrone and Viswanatha, 1997). Concentrations of DNA (plasmid or primer) were determined from the absorbance at 260 nm as follows: one absorbance unit corresponds to 50 and 38 μg/ml of the double-stranded (plasmid) and single-stranded (mutagenic primers) DNA, respectively (Sambrook *et al.*, 1989).

2.6. Determination of Lysine:N⁶-Hydroxylase Activity

The protocol employed for the measurement of lysine:N⁶-hydroxylase activity was similar to that reported previously (Parniak *et al.*, 1979). A typical assay, in a final volume of 5 ml, consisted of potassium phosphate (100 mM, pH 7.2), L-lysine (1 mM), FAD (40 μM), and *r*IucD (83.3 nM). The reaction was initiated by the introduction of an NADPH-generating system comprising $NADP^+$ (80 μM), glucose-6-phosphate (1 mM), and glucose-6-phosphate dehydrogenase (1.25 units) preincubated at 37°C for 5 min. Following incubation at 37°C for 15 min, the reaction was terminated by the addition of a slurry of Dowex 50W-X8 (200–400 mesh, H^+ form) resin in distilled water. The entire mixture was transferred to a 1.4 × 25-cm glass column and washed with 0.2 M HCl (40 ml) prior to elution with 6 M HCl (25 ml). The effluent was taken to dryness and the residue was dissolved in water and used for the determination of N⁶-hydroxylysine by the iodine oxidation procedure (Tomlinson *et al.*, 1971).

2.7. Proteolysis of *r*IucD

The influence of various ligands on *r*IucD's susceptibility to degradation by proteases was investigated both by an assessment of change(s) in its structural integrity by SDS–PAGE as well as in its ability to function as lysine monooxygenase. In these studies TPCK-trypsin, TLCK-chymotrypsin, and thermolysin served as representatives of endopeptidases, while carboxypeptidase Y served as an exopeptidase. The conditions chosen were such as to allow for the maintenance of *r*IucD in its native conformation and yet permit its rapid degradation upon treatment with the protease.

In the experiments with endopeptidases, the protocol employed was as follows: *r*IucD (10–14 μM) in 100 mM potassium phosphate, pH 7.0 (or 100 mM PIPES, pH 7.5, containing 10 mM $CaCl_2$, in the case of thermolysin), and endoprotease (5–7 μM); temperature, 25°C. Aliquots were drawn at desired intervals

and proteolysis was terminated by the addition of appropriate inhibitor (twofold excess of SBTI for TPCK-trypsin, 10 mM PMSF for TLCK-chymotrypsin, and 10 mM EDTA for thermolysin) prior to use for SDS–PAGE and determination of lysine:N^6-hydroxylase activity.

In the case of CPDY, a typical reaction mixture, in a final volume of 1 ml, comprised potassium phosphate (100 mM, pH 6.0), *r*lucD (10 μM), and CPDY (0.3 μM). The reaction was allowed to proceed at 37°C. Aliquots drawn at desired intervals were either diluted (1:1000) with phosphate buffer (pH 7.0) or with 200 mM tris-glycine (pH 8.3). The former sample was used for the determination of lysine:N^6-hydroxylase activity and the latter for SDS–PAGE analysis.

The final concentration of the cofactor or the substrate, when included, was as follows: FAD (0.5–1 mM), ADP (1 mM), NADPH (1 mM), and L-lysine (5 mM).

For the quantitative estimation of the amino acids released by action of exopeptidases, the procedure was similar to that documented previously (Martin *et al.*, 1977). Aliquots drawn at desired intervals were treated with a slurry of Dowex 50-X16 (H^+ form resin). The resin was washed extensively with water and the amino acids were recovered by elution with 6 N HCl. After removal of HCl, the sample was analyzed for amino acids by ion exchange chromatography (Spackman *et al.*, 1954).

In some experiments, the C-peptide (see Section 2.3 for details of the peptide preparation) was included in the reaction mixture over a concentration range of 0.1–0.5 mM.

2.8. Influence of FAD and ADP on the Catalytic Activity of Proteases

The effect of ADP and FAD on the proteolytic activity of endopeptidases (TPCK-trypsin, TLCK-chymotrypsin, and thermolysin) was assessed using azocasein as the substrate (Cowan and Daniel, 1982). A typical reaction, in a final volume of 3 ml, consisted of azocasein (1% w/v), potassium phosphate (100 mM, pH 7.5), and endopeptidase (150 μg). FAD and ADP when included were used at a final concentration of 1 mM. Following incubation at 25°C for 30 min, the reaction was terminated by the addition of an equal volume of TCA (10%). After 1 hr of standing at room temperature, the suspension was centrifuged and the extent of proteolysis was determined by measurement of the absorbance at 336 nm of the supernatant.

The enzymatic activity of CPDY was assessed using furyl acryloyl–L-Phe–L-Ala (FA–Phe–Ala) as substrate (Breddam, 1986). Typically, the reaction mixture, 1 ml in volume, consisted of potassium phosphate (100 mM, pH 6.0), FA–Phe–Ala (0.5–1.0 mM), and CPDY (10 μg). The assay, initiated by the addition of CPDY, was followed by monitoring the decrease in absorbance at 340 nm. When included in the experiment, the concentration of ADP or FAD was 1 mM.

The concentration of the C-peptide was varied over the range 0.06–0.5 mM in the experiments designed to assess its effect on the catalytic activity of CPDY.

2.9. SDS–PAGE and Western Blot Analysis

SDS–PAGE analyses of *r*lucD and its variants at various stages of their purification were performed using 10% polyacrylamide gels according to the procedure of Laemmli (1970).

Whole-cell lysates were also examined for the presence of the *iucD* gene product and these were prepared by suspension of cells, collected by centrifugation after 24 hr of growth, in SDS–PAGE sample buffer and boiled for 3 min.

For Western blot analysis, protein samples (25 μg protein), after separation by SDS–PAGE (12% gel), were transferred to nitrocellulose over 2 hr at 4°C followed by two brief washes in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5). Nonspecific binding of the antibody was inhibited by blocking the membrane with 5% (w/v) low-fat milk powder in TBS overnight at 4°C. The membrane was then rinsed twice with TTBS (TBS containing 0.05% Tween-20) for 5 min prior to incubation with purified polyclonal rabbit anti-*r*lucD (1:2000 dilution in TTBS) for 2 hr at room temperature. The membrane was rinsed twice in TTBS for 10 min at room temperature prior to incubation with goat anti-rabbit alkaline phosphatase conjugated secondary antibody (1:3030 dilution in TTBS) for 2 hr at room temperature. The membrane was subsequently washed twice for 5 min in TTBS and once in TBS for 5 min prior to detection by incubation with nitroblue tetrazolium and bromochloroindolyl phosphate (Blake *et al.*, 1984).

2.10. CD

CD spectra were recorded over the range 185–250 nm on a Jasco J-700 Spectropolarimeter set at 20 mdeg sensitivity, 0.2 nm resolution, four unit accumulation, 8 sec response, and a scanning speed of 200 nm/min. The *r*lucD concentration was 5–15 μM in 200 mM

potassium phosphate, pH 7.0. Measurements were made in a 300-μl cylindrical quartz cell with a 1 mm pathlength. Concentrations of ligands when used were FAD (200 μM), ADP (1 mM), and L-lysine (5 mM). All spectra were corrected for the appropriate blank solutions, recorded in the absence of enzyme. Each spectrum is the average of three scans. $[\theta]_{mr}$, the mean residue elipticity, is calculated using the following equation:

$$[\theta]_{mr} = \frac{CD}{10cln}$$

where l is the pathlength in cm, c is the molar concentration of the protein, n is the number of residues in the protein, and CD is the observable value.

2.11. DSC

Differential scanning calorimetric studies were performed using a Nano DSC from Calorimetry Sciences Corp. (Provo, UT). The *r*lucD concentration was 1 mg/ml in 200 mM potassium phosphate, pH 7.0, containing DTT (1 mM). Concentrations of ligands when present were FAD (0.5 mM), ADP (1 mM), and L-lysine (5 mM). Heating rate was 1 deg/min.

3. RESULTS

3.1. Stability of *r*IucD in the Absence of Cofactors and Substrate

Apo-*r*IucD preparations have been shown to undergo facile denaturation in media of low ionic strength. As a consequence, protein preparations are routinely maintained in media of ionic strength ≥0.25 and under these conditions *r*IucD exists in a tetrameric form with a molecular weight of approximately 200 kDa (Thariath *et al.,* 1993*a,b,* 1994). SDS–PAGE analyses of *r*IucD immediately after its isolation, under nonreducing conditions, have revealed the protein to migrate as a single component with a molecular weight (~50 kDa) corresponding to that of the monomer, indicating the absence of intersubunit disulfide bridges in its tetrameric structure. Storage of *r*IucD, either at 4°C or –80°C, under conditions that minimize its denaturation (potassium phosphate, pH 7.0, ionic strength ~0.25) has been found to be accompanied by a progressive loss in its monooxygenase activity. This phenomenon could be reversed with complete restoration of monooxygenase function by treatment with thiol reagents such as DTT (≥10 mM), indicating participation of the protein's cysteine residues in the formation of disulfide bridges during its storage. In order to assess whether these disulfide bridges occur within the tetramer (intratetramer) or between tetramers (intertetramer) of *r*IucD, the molecular weight of the protein was monitored by chromatography on Superdex 200 matrix at various intervals subsequent to its isolation. These studies have revealed that *r*IucD, immediately after isolation, exists predominantly as a tetramer (Fig. 1A). During storage, this form of the protein disappears with the concomitant appearance of species with molecular weights >200 kDa (Fig. 1B). Treatment of such protein preparations with DTT results in the restoration of the initial tetrameric state (Figs. 1C and 1D), indicating a rupture of intertetrameric disulfide bridges by the thiol reagent. As noted above, DTT treatment is also accompanied by restoration of the monooxygenase function of the protein. However, it is not possible to attribute the regeneration of enzymatic activity solely to the rupture of intertetramer disulfide bridges, since similar linkages occurring either within or between the monomeric subunits contributing to the tetrameric structure of the protein are also prone to reduction by the thiol reagents.

3.2. Interaction of *r*IucD with Its Ligands

The conformational changes in *r*IucD accompanying its interaction with its ligands were assessed by examining the CD spectra, DSC profiles of the protein, and its susceptibility to degradation by proteases. Neither the CD spectra nor the DSC profile of *r*IucD was significantly affected in the presence of its cofactors or substrate (Figs. 2 and 3). However, its susceptibility to the action of proteases was found to be influenced by the presence of some of its ligands; the details are given below.

Treatment of *r*IucD with TPCK-trypsin (see Section 2 for details) resulted in its rapid degradation of the protein with concomitant loss of its monooxygenase activity. Similar studies performed in the presence of FAD (0.5 mM) revealed the absence of such adverse action by the protease as indicated by the total retention of *r*IucD's structural integrity and catalytic function. Further investigations performed over a range of FAD concentrations indicated that a cofactor concentration of 200 μM was adequate to protect *r*IucD from proteolytic degradation. Interestingly, ADP (1 mM) was also found to shield *r*IucD from tryptic digestion as indicated by the preservation of the

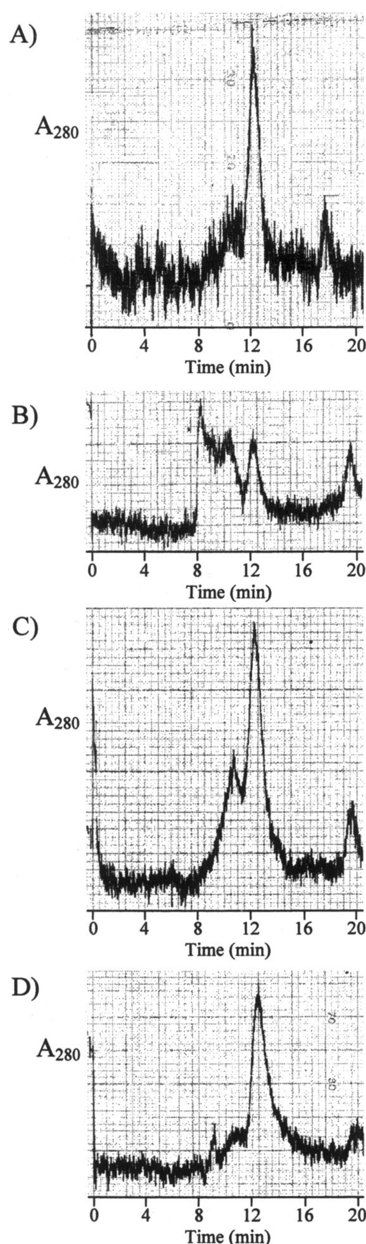

Fig. 1. Superdex 200 chromatography of *r*lucD preparations. The conversion of the tetrameric form of *r*lucD to polytetrameric species and the reversal of this process by DTT was monitored by chromatography of the samples on a 10 mm × 30 cm Superdex 200 column with 100 mM potassium phosphate, pH 7.0, serving both as equilibration and elution medium. Prior to chromatography of *r*lucD samples, calibration was achieved by the determination of the retention times of molecular weight standards as follows: cytochrome c (12.4 kDa), 17.6 min; carbonic anhydrase (30 kDa), 16.5 min; albumin (67 kDa), 14.1 min; alcohol dehydrogenase (128 kDa), 12.8 min; and β-amylase (200 kDa), 12.2 min. Under these conditions, freshly prepared *r*lucD was found to have a retention time of 12.4 min. Experimental conditions: protein concentration as shown; eluant, 100 mM potassium phosphate, pH 7.0; flow rate, 1 ml/min; chart speed, 0.4 cm/min. (A) *r*lucD immediately after isolation from chromatography on Orange A Dye matrix (Thariath *et al.*, 1993a), 50 μl (8 μg) of sample was injected; (B) *r*lucD sample from storage at −80°C, allowed to thaw at 4°C for 24 h (free of NaCl and DTT); 25 μl (34 μg) of sample was injected; (C) *r*lucD, same as in B, but for treatment with DTT (10 mM) for 75 min; 25 μl of sample was injected; (D) *r*lucD, same as in B, but for treatment with DTT (50 mM) for 180 min; 25 μl of sample was injected. Species emerging at 12.4 min represents the *r*lucD tetramer; species emerging at times <12.4 min reflect the polytetrameric form(s) of *r*lucD; and the species emerging at times >12.4 min represent buffer ions and/or DTT.

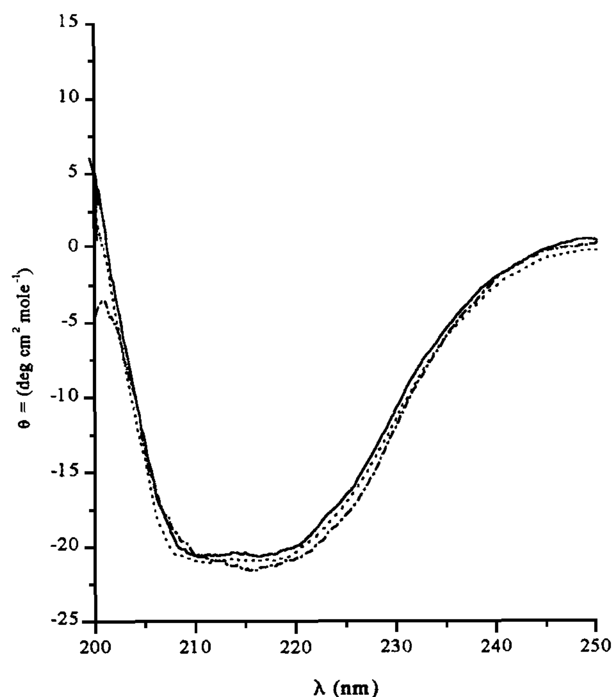

Fig. 2. CD spectra of *r*lucD. Solid line, no ligands; dashed line, lysine (1 mM); dashed and dotted line, lysine (1 mM) and FAD (200 μM).

protein's structural integrity and catalytic function. Of the other two obligatory participants in the catalytic process mediated by *r*lucD, NADPH and L-lysine, the former was partially effective, while the latter (5 mM) was totally ineffective in preventing the protein from degradation by trypsin. Thus, in the presence of NADPH (1 mM), approximately 50% *r*lucD was degraded by the protease as indicated by SDS–PAGE analysis and measurement of lysine:N^6-hydroxylase activity. These results are presented in Fig. 4.

In the experiments with TLCK-chymotrypsin and thermolysin, the observations were similar to those recorded in the studies with TPCK-trypsin. Both FAD and ADP prevented *r*lucD degradation by these proteases, while NADPH and lysine were marginally effective and ineffective respectively (data not shown).

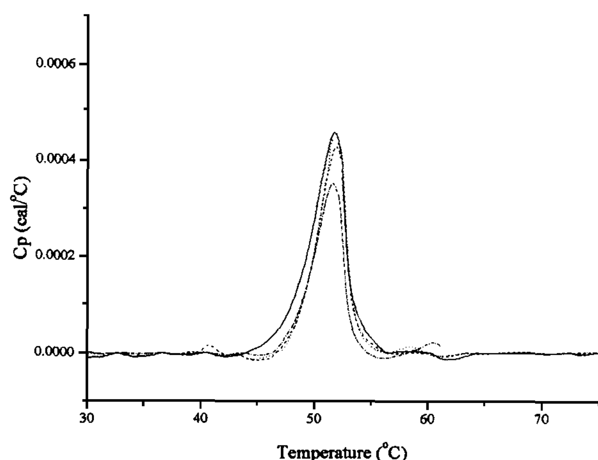

Fig. 3. DSC profile of *r*IucD. Solid line, no ligands; dashed line, ADP (1 mM); dotted line, FAD (0.5 mM); dashed and dotted line, lysine (5 mM).

The protective effect exerted by FAD and ADP does not appear to be due to their ability to function as inhibitors of the proteases since these compounds have no adverse effect in experiments with azocasein serving as a substrate.

The reaction of *r*IucD with CPDY was accompanied by a steady decline in lysine:N^6-hydroxylase activity with approximately 85% activity being lost after 80 min of incubation (Fig. 5). At this stage of the reaction with CPDY, analyses revealed the release of the following C-terminal amino acids residues from *r*IucD (amounts relative to that of threonine in parentheses): Thr (1.00), Ser (1.20), Gly (1.80), Arg (0.91), Glu (0.25), Ile (0.80), Leu (0.96), Ala (1.10), and Pro (1.20). Allowing for the loss of tryptophan under strong acidic conditions and the low recovery of glutamic acid, due to its poor retention by the cation exchange resin (in view of the relatively high ionic strength of the CPDY reaction medium), the above data would appear to be consistent with the deletion of 10–11 C-terminal amino acid residues from *r*IucD. Interestingly, both FAD (1 mM) and ADP (1 mM) offered protection from such deleterious effects as indicated by total retention of *r*IucD's monooxygenase activity even after prolonged exposure to CPDY, while NADPH and lysine were devoid of such beneficial action (Table II). Finally, FAD and ADP were found to have no adverse effect on the ability of CPDY to mediate the hydrolysis of its typical peptide substrate, furylacryloyl–Phe–Ala. Hence, their protective influence observed in the studies with *r*IucD would appear to be due to factors other than functioning as inhibitors of the exoprotease.

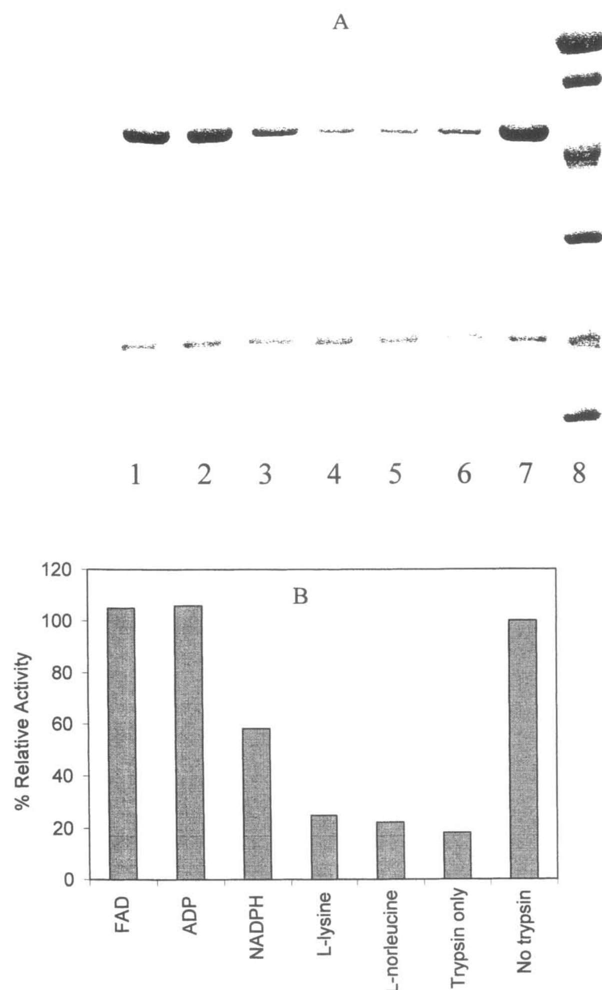

Fig. 4. (A) SDS–PAGE profile of *r*IucD samples treated with TPCK-trypsin. *r*IucD (14 μM) was incubated (25°C, 12 min) with TPCK-trypsin (7 μM) in the presence of cofactors and substrate indicated. The reaction was stopped by the addition of SBTI (15 μM) prior to analysis. Lanes: 1, FAD (0.5 mM); 2, ADP (1.0 mM); 3, NADPH (1.0 mM); 4 L-lysine (5 mM); 5, L-norleucine (1 mM); 6, none; 7, control, trypsin added after SBTI; 8, molecular weight standards expressed in kDa (phosphorylase b, 94; serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; trypsin inhibitor, 20; and α-lactalbumin, 14). (B) Vertical bar graph representation of lysine:N^6-hydroxlyase activity following treatment with TPCK-trypsin. Lysine:N^6-hydroxlyase activity was determined after addition of SBTI as mentioned above in A. The vertical bars are matched to their corresponding lanes in A. Increase in NADPH concentration has been found to result in an enhanced protection from degradation by the protease, with maximum value (~80%) being achieved at an NADPH concentration of 7 mM (data not shown). Studies with azocasein as substrate revealed that none of the cofactors, even at the highest concentrations, function as inhibitors of the proteases.

The deletion of the C-terminal residues of *r*IucD would appear to result in a disruption of its native conformation as indicated by the development of

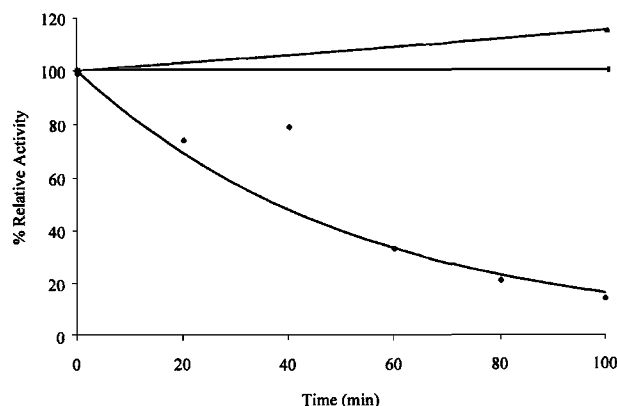

Fig. 5. Effect of CPDY on lysine:N^6-hydroxylase activity of *r*lucD. *r*lucD (10 μM) in 100 mM potassium phosphate, pH 6.0, was treated with CPDY (20 μg) at 37°C. The concentration of other compounds, when included, were FAD (0.5 mM) and ADP (1.0 mM). Aliquots taken at desired intervals were diluted (1:1000) with 200 mM potassium phosphate buffer, pH 7.0, to stop further degradation and used for the determination of lysine:N^6-hydroxylase activity.(♦-♦) none;(■-■) control (no CPDY); (▲-▲)FAD or ADP. L-Lysine (5 mM) failed to protect from the adverse action of CPDY (data not shown).

Table II. Susceptibility of *r*lucD to Degradation by Proteases: Influence of Substrate, Cofactors, and Analogs[a]

	Percentage relative activity		
Effector	TPCK-trypsin	TLCK-chymotrypsin	Carboxypeptidase Y
FAD (0.5 mM)	105	107	139
ADP (1.0 mM)	106	114	114
NADPH (1.0 mM)	58	n.d.	7
L-Lysine (5 mM)	25	4	11
None	18	4	8
Control	100	100	100

[a] In the case of TPCK-trypsin and TLCK-chymotrypsin, *r*lucD (12–14 μM) in 100 mM potassium phosphate, pH 7.0, was treated with the protease (6–7 μM) at 25°C. After 12 min, the reaction was stopped by the addition of SBTI or PMSF prior to determination of lysine:N^6-hydroxylase activity. In the case of CPDY, *r*lucD (10 μM) in 100 mM potassium phosphate, pH 6.0, was treated with the exopeptidase (0.3 μM) for 2 hr at 37°C prior to determination of lysine: N^6-hydroxylase activity. The concentration of effector, when included, is as shown in the table. Similar results were obtained with thermolysin (data not shown).

opalescence in the reaction mixture(s) after prolonged (≥75 min) incubation with the exoprotease. Since these observations suggested a crucial role for the C-terminal segment in the maintenance of *r*lucD in its native conformation, the possibility of synthetic peptide mimic(s) compensating for the section deleted by CPDY was explored by performing experiments in the presence of the C-peptide (Pro-Ala-Leu-Ile-Gln-Trp-Arg-Ser-Gly-(D)-Thr; see Section 2 for details). These studies showed that in the presence of the C-peptide (0.5 mM), *r*lucD's monooxygenase activity remained unaffected even after prolonged (≥ 3 hr) period of reaction with CPDY. Hence, the following three aspects were considered to explain the protective influence exerted by the C-peptide: (i) its ability to compensate for the section of the protein deleted by CPDY; (ii) the possibility of its competing with *r*lucD and serving as a substrate for CPDY; and (iii) its capacity to serve as an inhibitor of CPDY. The first of these three aspects would appear untenable, since addition of C-peptide to *r*lucD preparations pretreated with the CPDY did not result in the restoration of lysine monooxygenase activity. Concerning the second aspect, HPLC analysis of the C-peptide preparation subsequent to its treatment with CPDY indicated the absence of any degradation, pointing to its inability to serve as a substrate for the exoprotease. This finding is not unexpected in view of the D-configuration of the C-peptide's carboxy terminal residue, a feature deliberately incorporated to minimize its serving as a substrate for CPDY. The ability of the C-peptide to act as an inhibitor of CPDY was confirmed in the studies using furylacryloyl–Phe–Ala. Data shown in Fig. 6 show that the C-peptide is an effective inhibitor of CPDY, with approximately 90% inhibition in the hydrolysis of the chromophoric peptide substrate being noted when it is present at a concentration of 90 μM.

3.3. Studies with C-Terminal-Truncated Forms of *r*lucD

In order to gain unambiguous insight into structural feature(s) indispensable for the maintenance of

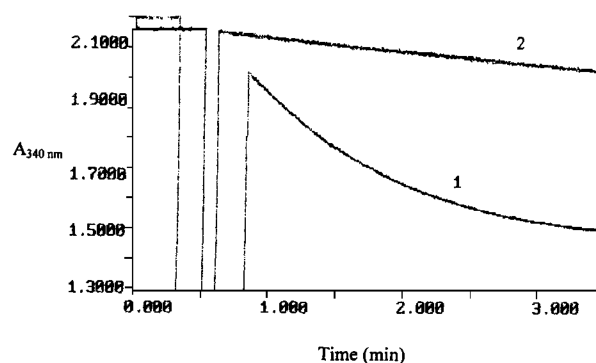

Fig. 6. CPDY-catalyzed hydrolysis of furylacryloyl–Phe–Ala. Conditions: FA–Phe–Ala (1 mM) in 100 mM potassium phosphate, pH 6.0, with CPDY (10 μg). (1) In the absence of C-peptide; (2) in the presence of C-peptide (90 μM).

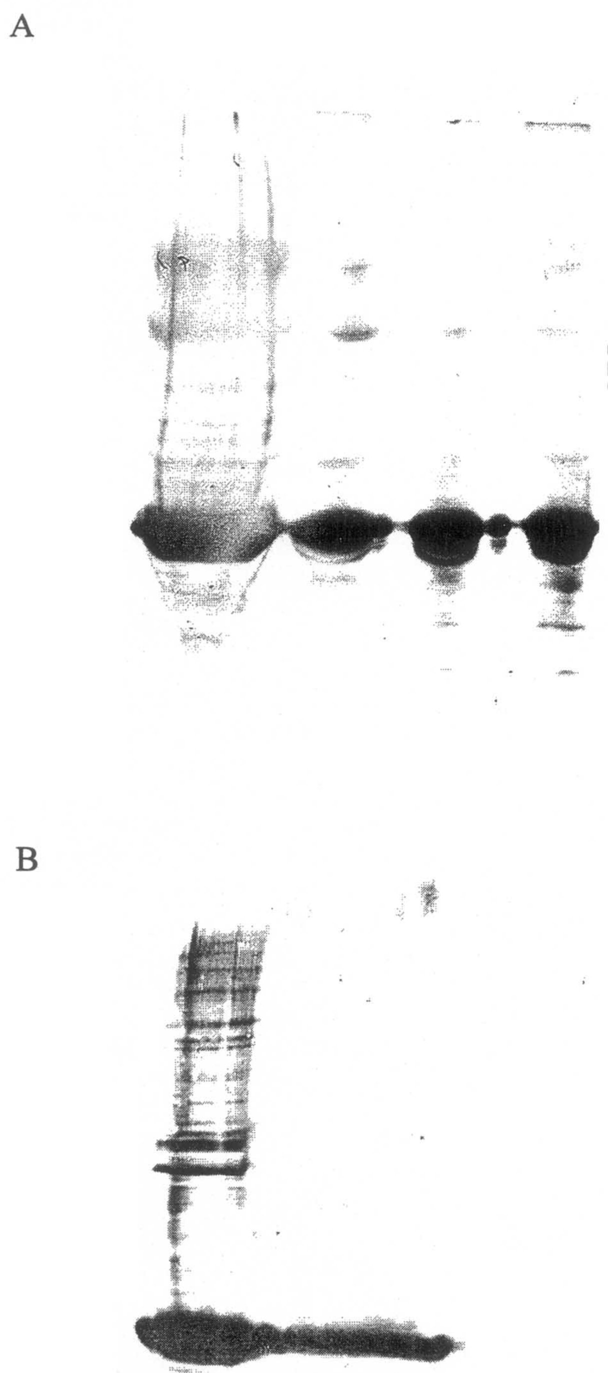

Fig. 7. Western blot analyses. The expression of truncated forms of *r*IucD in *E. coli* DH5α and their stability during the protocol for their purification was monitored by Western blot analyses using polyclonal rabbit anti-*r*IucD antibodies (see Section 2 for details). Results obtained with *r*IucD Δ17 are typical of those recorded with other truncated *r*IucD preparations. Similar studies with intact *r*IucD served as a control. (A) Western blot identification of *r*IucD, from left to right: (i) whole-cell lysate, (ii) crude cell-free extract, (iii) ammonium sulfate (40%) fraction, and (iv) pure *r*IucD. (B) Western blot identification of *r*IucD Δ17, from left to right: (i) whole-cell lysate, (ii) cell-free extract, and (iii) ammonium sulfate (40%) precipitate.

*r*IucD in its native conformation, four well-defined *r*IucD variants, differing in the extent of C-terminal deletions, were developed (Table I). The ability of truncated versions of *iucD* to encode for a catalytically functional protein was assessed by examining the culture fluids for the presence of N^6-hydroxylysine, an inherent feature of cells expressing the catalytically functional form of *r*IucD. Such analyses revealed the presence of N^6-hydroxylysine only in the culture fluid of cells designed to express *r*IucD Δ8 and not in the case of cells transformed to express the other three truncated *iucD* gene products, namely *r*IucD Δ17, *r*IucD Δ14, and *r*IucD Δ11. The absence of N^6-hydroxylysine in the case of the latter three cultures could be either due to their inability to express these particular truncated forms of *iucD* or a reflection of a loss of catalytic function in the three truncated forms of *r*IucD. Analyses of the whole-cell lysates of cultures expressing these truncated proteins revealed the presence, in all four cases, of a protein with a molecular weight corresponding to that of the *iucD* gene product, and its identity as such was further confirmed by Western blot analyses using polyclonal antibodies raised against *r*IucD (Fig. 7). Thus, all four truncated forms of the *iucD* gene product would appear to be expressed. However, while *r*IucD Δ8 is capable of lysine N-hydroxylation, the other three muteins (*r*IucD Δ17, *r*IucD Δ14, and *r*IucD Δ11) are devoid of such catalytic activity. Furthermore, during attempts to isolate these truncated *r*IucD preparations, it was noted that their presence could be detected only in the whole-cell lystates and the crude cell-free systems, but not in the fractions obtained in subsequent stages of purification. This finding is in distinct contrast with those noted in the case of normal *r*IucD, which is detectable during all stages of purification (Thariath *et al.,* 1993*a, b,* 1994): The lability of these truncated *r*IucD preparations during later stages of purification would appear to be related to the removal of flavin cofactor(s) normally associated with whole cells and the crude cell-free system. This observation is consistent with the protective action of FAD mentioned in the previous section.

4. DISCUSSION

The observations recorded in this study have revealed that neither the substrate (L-lysine) nor the co-

factor (FAD) elicits significant conformational changes in *r*IucD as is evident from the CD and DSC profiles (Figs. 2 and 3). However, when susceptibility to degradation by proteases is employed as a criterion for monitoring the structural changes in *r*IucD, significant differences in the response due to interaction with its various ligands become apparent. Thus, *r*IucD, in the absence of its cofactors or substrate, is susceptible to degradation both by endopeptidases as well as an exopeptidase (CPDY) with concomitant loss of its monooxygenase function. Such adverse action of proteases on *r*IucD is completely prevented when experiments are performed in the presence of low concentrations of FAD (0.2 mM) or ADP (0.5 mM). Concerning the other two ligands, NADPH (1 mM) has been found to confer partial protection, while the substrate, L-lysine (5 mM), is virtually ineffective in preventing the proteolytic degradation of *r*IucD. The reasons for the inability of L-lysine, the preferred substrate, to protect *r*IucD from the deleterious action of proteases are not clear. In the case of the flavin cofactor, the magnitude of the dissociation constant K_D of the *r*IucD–ligand complex would appear to govern their effectiveness in rendering the protein resistant to the action of proteases. Thus, since *r*IucD's interaction with FAD is characterized by a K_D value of approximately 20 μM (Marrone, 1997), cofactor concentrations (≥200 μM) that ensure the maintenance of the protein in the ligand bound, or the holoenzyme, state are effective in protecting it from proteolytic degradation. The ability of ADP to bind at the flavin cofactor site (Marrone, 1997) appears to be responsible for its protective effect.

Under conditions identical with those employed in the studies with FAD, NADPH (1 mM) has been found to be only partially effective in preventing the proteolysis of *r*IucD as indicated by the retention of ~50% of the protein's monooxygenase activity (Fig. 4). Further increases in the concentration of NADPH result in an enhancement in the degree of protection afforded to *r*IucD, with a maximum value, ~80% retention of monooxygenase activity, being observed at cofactor concentrations ≥7 mM (data not shown). The reasons for not achieving complete protection are not obvious. The finding may be a reflection of a high K_D value of the *r*IucD–NADPH complex. Although an apparent K_M value of approximately 100 μM for the reduced pyridine nucleotide cofactor has been recorded in experiments with lysine:N^6-hydroxylase activity serving as the criterion for the assessment (Thariath *et al.*, 1993*b*, 1994), efforts to determine the actual K_D value of the *r*IucD:NADPH complex have been futile. It is not clear whether this situation is a reflection of the protein's binding of NADPH being contingent upon its initial interaction with FAD. The situation appears to be similar to that recorded in the case of *p*-hydroxybenzoate hydroxylase, which also utilizes FAD and NADPH as cofactors. In the case of this latter enzyme, X-ray crystallographic studies have not been successful in identifying the NADPH-binding domain, in view of the cofactor's propensity to displace FAD from its binding domain in the protein (van der Laan *et al.*, 1989). These observations pertaining to the protection of *r*IucD by ligands are analogous to those recorded in the case of glucose oxidase (Swoboda, 1969), creatine kinase (Jacobs and Cunningham, 1968), and NADPH-quinone reductase (Chen *et al.*, 1994).

Both the studies with CPDY as well as those with well-defined C-terminal deletion variants of *r*IucD have provided unambiguous evidence for the indispensability of the C-terminal segment for *r*IucD to function as a monooxygenase. The latter approach has also resulted in pinpointing the segment essential for the protein to be catalytically functional. Thus, while *r*IucD Δ11 is incapable of N^6-hydroxylysine production, the *r*IucD Δ8 variant is competent to perform such a function. This observation suggests that the structural feature(s) inherent in this segment, residues 415–417, may be crucial for the maintenance of *r*IucD in its catalytically active conformation.

Reports concerning the importance of the C-terminal domain for the structural integrity and/or catalytic function of proteins have been widely documented (Yu *et al.*, 1998; Dai and Wang, 1997; Albalat *et al.*, 1995; Walter *et al.*, 1999; Nechushtan *et al.*, 1999; Shimada *et al.*, 1999; Criado *et al.*, 1999; Mata *et al.*, 1999; Allardyce *et al.*, 1999). The observations recorded in the case of lipoamide dehydrogenase from *Azotobacter vinelandii* would appear to be especially relevant since this protein, like *r*IucD, is both oligomeric and utilizes FAD for its catalytic function (Mattevi *et al.*, 1991). The function of the C-terminus of this protein has been investigated by deletion of 5, 9, and 14 C-terminal residues respectively. Deletion of 5 residues had no adverse effects, that of 9 residues led to decreased conformational stability, and the removal of 14 residues was accompanied by complete loss of catalytic function (Benen *et al.*, 1992). Furthermore, these studies also demonstrated the presence of intersubunit interaction involving a tyrosine residue at the N-terminus of one subunit and a histidine at the C-terminus of the second subunit. This structural feature, which is proposed to be involved in mediating the redox properties of the flavin cofactor via the conformation of the C-terminal segment, is not present in preparations of en-

zyme with deletion of either 9 or 14 C-terminal residues (Benen *et al.,* 1992). Further studies are needed, especially the elucidation of the three-dimensional structure of the protein, to determine whether a similar situation as above prevails in *r*IucD.

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