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Lysine : N^6-Hydroxylase: Cofactor Interactions

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Recombinant lysine : N^6-hydroxylase (rIucD) requires the cofactors FAD and NADPH for its catalytic function of the conversion of L-lysine to its N^6-hydroxy derivative. In the presence of high concentration of chloride ions ($\geq$ 600 mM), the protein exists in a reversible inactive conformation. Depending on the oxidation state of its thiol functions, rIucD can bind 2,6-dichlorophenol indophenol (DPIP), either covalently or noncovalently, the former type of interaction occurring with protein preparations possessing unmodified thiol groups. Both covalent and noncovalent complexes of rIucD and DPIP appear to be capable of NADPH oxidation in the presence of exogenous DPIP by a phenomenon of exchange of reducing equivalents between the protein-bound dye and that free in the medium. In the presence of FAD, the latter type of complex has been found to function as a diaphorase. The diminution in the catalytic activity of rIucD observed at high concentrations of the flavin cofactor does not appear to be due to an uncoupling of the processes of NADPH oxidation and lysine : N-hydroxylation caused by an exchange of reducing equivalents between the enzyme-bound FAD and that free in the medium.

INTRODUCTION

Lysine : N^6-hydroxylase (IucD)[2] catalyzes the conversion of L-lysine to its N^6-hydroxy derivative, the initial step in the biosynthesis of aerobactin, a siderophore that functions as a virulence determinant in many septicemic organisms (*1–4*). This enzyme normally occurs in a membrane environment, a feature that has precluded characterization of both its physicochemical properties and its catalytic function (*5, 6*). Recently, a gene fusion approach has led to the production of cytoplasmic rIucD preparations, and two of these rIucD439 and rIucD398, have been isolated as apoproteins (*7, 8*). These rIucD preparations catalyze the conversion of L-lysine to its N^6-hydroxy derivative on supplementation with NADPH and FAD, with the process of NADPH oxidation being coupled to that of lysine : N-hydroxylation. In

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[2] Abbreviations used: rIucD, recombinant cytoplasmic lysine : N^6-hydroxylase; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); DPIP, 2,6-dichlorophenol indophenol; G-6-P, glucose 6-phosphate; G-6-P deH_2, glucose-6-phosphate dehydrogenase; DTT, dithiothreitol; ESMS, electrospray mass spectroscopy; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; BSA, bovine serum albumin; ADPR, 5′-adenosine diphosphate ribose; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis.

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the absence of hydroxylatable substrate, rIucD-mediated oxidation of NADPH is slow and the process is channeled toward H_2O_2 production (*8*). Current studies concern further characterization of rIucD and its electron transfer reactions.

MATERIALS AND METHODS

FAD, $NADP^+$, NADPH, DTNB, NTCB, iodoacetate, G-6-P, G-6-P dehydrogenase, DTT, and mercaptoethanol were purchased from Sigma (St. Louis, MO). DPIP and guanidine hydrochloride (ultrapure) were obtained from J. T. Baker (Phillipsburg, NJ). [^{14}C]Iodoacetate was purchased from Amersham Life Science Inc. (Arlington Heights, IL). Dyemātrex Orange A affinity gel was purchased from Amicon (Beverly, MA). Bio-Gel P4 was obtained from Bio-Rad (Richmond, CA).

The isolation and purification of lysine: N^6-hydroxylase (rIucD439) were achieved by employing procedures documented in the literature (*7, 8*). The concentration of rIucD was determined spectrophotometrically using an ε_m value of 6.75×10^4 M^{-1} cm^{-1}.

Determination of Enzymatic Activity

The protocol employed for the measurement of lysine: N^6-hydroxylase activity was similar to that reported previously (*9*). 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), $NADP^+$ (80 μM), G-6-P (1 mM), G-6-P dehydrogenase (1.25 units), and rIucD (83.3 nM). 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 1.2×25-cm glass column and washed with HCl (40 ml, 0.2 N) prior to elution with 25 ml of HCl (6 N). The effluent was taken to dryness and the residue was dissolved in water and used for the determination of N^6-hydroxylysine by the iodine oxidation procedure (*10*).

Determination of H_2O_2

The procedure described by Hildebrandt *et al.* (*11*) was used. In a typical experiment, the assay mixture in a final volume of 20 ml consisting of NADPH (200 μM), FAD (33 μM), potassium phosphate (100 mM, pH 7.2), and rIucD (83.3 nM) was incubated at 37°C. At desired intervals, an aliquot (1.5 ml) was removed and treated with an equal volume of trichloroacetic acid (3%). The mixture was centrifuged (5000*g*, 5 min) and the clear supernatant was treated with ferrous ammonium sulfate (0.5 ml, 10 mM), followed by potassium thiocyanate (0.2 ml, 2.5 M). After 10 min at room temperature, the absorbance at 480 nm was recorded. This analytical procedure is not influenced by the presence of NADPH, which has been found to interfere in the alternative method of estimation involving the use of peroxidase (*12*).

Determination of Sulfhydryl Groups

The thiol content of rIucD was determined by titration with DTNB (*13*) either under native or under denaturing conditions. In a typical experiment, a solution of rIucD (1 ml, 7–12 μM) in potassium phosphate (200 mM, pH 7.6) was treated with DTNB (100 μl, 10 mM), and the increase in absorbance at 412 nm was recorded. An ε_M value of 14,150 M^{-1} cm^{-1} (*14*) was used to quantitate the number of thiol groups in the protein. Similar experiments performed in the presence of guanidine hydrochloride (4 M) provided an estimate of the number of thiol groups in the denatured rIucD preparation.

In some instances, the thiol content of rIucD was determined by titration with NTCB (*15*). The experimental conditions were identical to those described above except for the replacement of DTNB with NTCB.

Alkylation of rIucD

The cysteine content of rIucD was assessed by alkylation with [^{14}C]iodoacetate. In a typical experiment, rIucD (40 nmole) in potassium phosphate (200 mM, pH 7.0) containing guanidine hydrochloride (4 M) was treated with an aliquot of an aqueous solution of [^{14}C]iodoacetate to achieve a 20-fold molar excess of the reagent over that of the cysteine residues present in the protein. After 20 min of incubation, the reaction was terminated by the addition of DTT (equimolar to that of iodoacetate). The reaction mixture was dialyzed extensively against distilled water. The protein (which was precipitated on dialysis) was collected and dissolved in guanidine hydrochloride (4 M) prior to assessment of radioactive label with the aid of a Beckman Model 5000TD liquid scintillation counter. The number of cysteine residues in rIucD was determined from the radioactive label incorporated into the protein.

Isolation of Covalent DPIP–rIucD Complex

rIucD (20 nmol) in 1 ml (200 mM potassium phosphate, pH 7.0) was treated with an aliquot of an aqueous solution of DPIP (1 mM) to achieve a final concentration of 100 μM with respect to the reagent. After 10 min of standing at 25°C, the DPIP–rIucD complex was recovered by chromatography on 1 × 10-cm column of Bio-Gel P4 with 200 mM potassium phosphate, pH 7.0, serving as both an equilibration medium and an elution medium.

Preparation of 2-Mercaptoethanol–DPIP Conjugate

Ten milliliters of DPIP (10 mM) in 30 mM potassium phosphate, pH 7.0, was treated with an aliquot of 2-mercaptoethanol to achieve a final concentration of 20 mM with respect to the reagent. Addition of the thiol agent resulted in a rapid bleaching of the dye due to its conjugation with the thiol functions (*16–18*); however, on standing, the reaction mixture turned blue presumably due to the oxidation of the conjugate. After 12 h of standing, the reaction mixture was diluted to 100 ml. This preparation served as the source of 2-mercaptoethanol–DPIP conjugate used in these investigations.

RESULTS

Characteristics of rIucD Preparation

The recombinant lysine: N^6-hydroxylase preparations (rIucD439), used in the current investigation should, in the absence of post-translational processing, comprise 439 amino acid residues, 13 of which are contributed by the β-galactosidase fragment and the rest are those of the *iucD* gene product (*7*). ESMS analyses of these preparations (homogeneous by SDS–PAGE) reveal a molecular weight of 48815 $\pm$ 5 Da. Since the primary translation product, rIucD439, is expected to have a molecular weight of 50,270, the observed lower value would suggest that the protein has been processed by a post-translational mechanism. The occurrence of such a phenomenon, which leads to the deletion of the β-galactosidase peptide segment from rIucD439, has been confirmed by analysis of the *N*-terminal amino acid sequence of the protein (*19*). Removal of 14 amino acid residues from the N terminus of rIucD439 by a post-translational processing event would be compatible with the observed molecular weight for the rIucD preparation.

The final step in the purification of rIucD involves chromatography on Dyemātrex Orange A, and the lysine: N^6-hydroxylase activity is usually recovered in the fractions eluted with buffer containing high concentrations of NaCl ($\geq$750 mM). Although rIucD has been found to be capable of its normal catalytic function of lysine: N^6-hydroxylation at concentrations of Cl^- ions $\leq$50 mM (a situation that prevails under assay conditions), a comprehensive study of the effect of these anions was initiated especially in view of their adverse action on a number of flavin-dependent monooxygenases (*20–23*). Such information may prove useful in identifying the conditions that would promote the maintenance of rIucD in its native conformation, a prerequisite for the production of the crystals for the elucidation of the three-dimensional structure of the protein. For these experiments, the enzyme preparations were rendered free of chloride ions by chromatography on a 1 $\times$ 10-cm column of Bio-Gel P4 with 200 mM potassium phosphate, pH 7.0, serving as both equilibration and elution medium. The enzymatic activity was assessed as a function of increasing concentration of chloride ions in the assay. These results were compared with those obtained in the experiments performed in the presence of either phosphate or sulfate ions under conditions of identical ionic strength. As shown in Fig. 1, the enzymatic activity of rIucD is not influenced by either phosphate or sulfate ions. In contrast, the catalytic activity of rIucD declines steadily with increasing concentration of Cl^- ions in the assay, with almost complete loss of activity noted at a 600 mM concentration of the anion (Fig. 1). Since enzymatic activity is regained on dilution (to 50 mM or less), rIucD appears to be in a reversible, inactive conformation in a medium high in NaCl concentration ($\geq$600 mM). The destabilizing influence of Cl^- ions is further indicated by the observation that rIucD in a medium containing NaCl ($\geq$600 mM) loses >75% of its enzymatic activity on freezing in liquid nitrogen, whereas similar treatment performed in the presence of phosphate ions ($\approx$200 mM) results in the retention of >70% catalytic function.

During storage in a medium of high ionic strength $\geq$0.25, pH 7.0, the enzyme preparation undergoes loss in enzymatic activity that can be restored by treatment

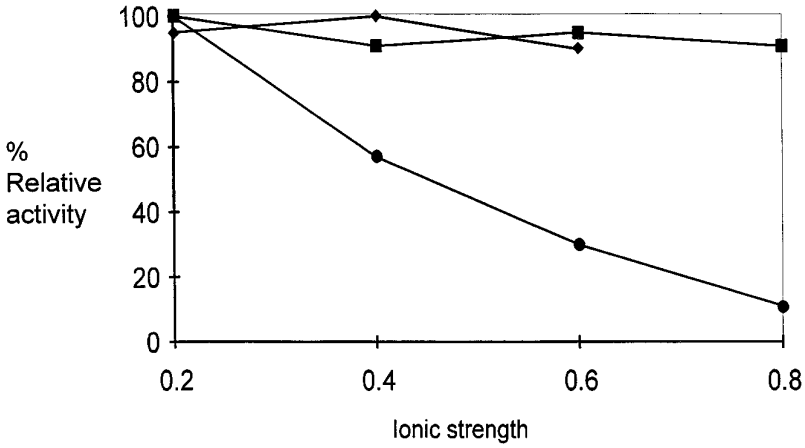

FIG. 1. Influence of various anions for similar ionic strengths on the lysine : *N*-hydroxylase activity of rIucD: Assays for lysine : *N*-hydroxylase activity were performed at pH 7.0 (see text for details) in the presence of various anions as indicated. ◆, Phosphate ions; ■, sulfate ions; ●, sodium chloride.

with thiols like DTT (1 mM). This observation, taken together with the inactivation of the enzyme by thiol modifying agents (*7, 8*), suggests that these function(s) may play an indispensable role in rIucD's catalytic mechanism. Two approaches were employed to determine the number of free thiol functions in the rIucD preparation. The first pertained to the titration by DTNB (*13*) or NTCB (*15*). Treatment of the native protein preparation with either DTNB or NTCB indicated the presence of three titratable thiol functions per rIucD monomer. Of these, two thiol groups reacted rapidly with DTNB while the reaction of the third one was slow, requiring approximately 10 min for completion of the reaction. Repetition of the experiment in the presence of guanidine hydrochloride (4 M) revealed the presence of approximately five DTNB-titratable thiol groups per rIucD monomer. These results are presented in Table 1. Similar results were obtained when experiments were performed with rIucD preparations exposed to high concentrations of NaCl, i.e., the presence of three DTNB titratable thiol functions per rIucD monomer in the native conformation and five such functions on denaturation of the protein.

Alkylation of the protein with [^{14}C]iodoacetate in the presence of guanidine hydrochloride, pH 7.0, revealed the incorporation of approximately 4.8 ± 0.3 mol/mol of rIucD monomer. These observations are consistent with the presence of five cysteine thiol functions per rIucD monomer. Thus, of the six cysteine residues reported to be present in the *iucD* gene product (*24*), only five are amenable to modification by either DTNB or iodoacetate. Details regarding the relative reactivities of the various thiol functions will be presented elsewhere.

Reaction of rIucD with DPIP

DPIP in 100 mM potassium phosphate, pH 7.0, absorbs maximally at 600 nm and there is no detectable change in absorbance over an extended period. On addition of rIucD, however, the absorbance at 600 nm declines steadily, reaching a constant

TABLE 1
DTNB-Titratable Thiol Groups in rIucD Preparations[a]

Sample	Number of thiol functions per rIucD monomer	
	Native	Denatured
rIucD	3.04 ± 0.15	5.05 ± 0.27
DPIP–rIucD	n.d.	2.89 ± 0.28

[a] The thiol content of rIucD preparations was determined both in the native and in the denatured (4.0 M guanidine · HCl) state by titration with DTNB (see text for details). Values represent averages of determinations, in duplicate, on three different rIucD samples prepared during this study. n.d., not determined.

value after approximately 5 min, and the magnitude of this decrease in absorbance value is proportional to the concentration of rIucD. On chromatography of the reaction mixture on Bio-Gel P4 matrix, the protein is recovered as a blue-colored complex with the dye, which cannot be removed by extraction with ethanol. These observations are consistent with a covalent modification of rIucD by DPIP. The DPIP–rIucD complex is characterized, in addition to its typical uv absorption band at 280 nm, by a visible absorption band with a λ_{max} value at 654 nm. This observed red shift in λ_{max} of the dye appears to be specific to its interaction with rIucD since DPIP bound to BSA retains its typical spectral features (Fig. 2, inset). Calculations based on the ε_m values of 6.75×10^4 and 2×10^4 M^{-1} cm^{-1} for the protein and the dye, respectively, suggest the incorporation of 1 mole of DPIP per rIucD monomer, and the dye in the complex appears to be distributed equally between its reduced and oxidized states.

Since DPIP has been shown to conjugate with mercaptans (*16–18*), the thiol content of rIucD was assessed by titration with DTNB or NTCB both prior to and after its interaction with DPIP. As noted before, rIucD preparations used in this investigations are characterized by the presence of five DTNB (or NTCB)-titratable functions per monomer; three of these are accessible in the native conformation, and the remaining two become susceptible to modification only on denaturation of the protein. Analyses of the DPIP–rIucD complex revealed the presence of approximately three DTNB-titrable groups per rIucD monomer under denaturing conditions (Table 1). These observations indicate that two of the thiol functions present in the native conformation of rIucD are participating in the oxidative substitution of DPIP, the phenomenon being analogous to that recorded with mercaptans (*16–18*). This view is consistent with the observation that modification of rIucD by DTNB results in a loss in its ability to form a covalent conjugate with DPIP.

The conjugation of rIucD with DPIP is accompanied by a loss in its ability to catalyze *N*-hydroxylation of lysine when assayed in the presence of FAD and NADPH cofactors normally required for this function of the protein; however, DPIP–rIucD complex either isolated or generated *in situ* is capable of promoting

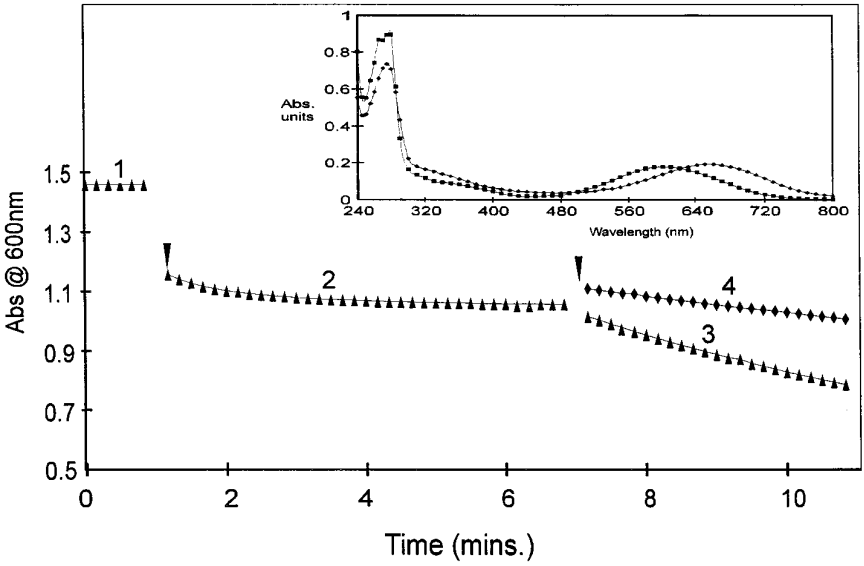

FIG. 2. Spectral and catalytic properties of DPIP–rIucD complex. DPIP–rIucD complex was prepared by treatment of rIucD (15 nmole) with DPIP (100 μM) in potassium phosphate (200 mM, pH 7.0) and was isolated by chromatography on Bio-Gel P4 (see text). For comparison, DPIP–BSA complex was also produced by a similar procedure. Spectra of DPIP–rIucD complex (◆) and DPIP–BSA complex (■) are shown in the inset. The main figure illustrates the ability of DPIP–rIucD complex to catalyze NADPH-dependent reduction of exogenous DPIP. **1,** DPIP in potassium phosphate (100 mM, pH 7.0); **2,** reaction with rIucD leading to the formation of DPIP–rIucD complex; **3,** NADPH-dependent reduction of exogenous DPIP mediated by the DPIP–rIucD complex. The introduction of rIucD and NADPH is indicated by the first and second arrows, respectively. **4,** Reaction of DPIP with NADPH in the absence of DPIP–rIucD complex shown for comparison. The DPIP–rIucD complex recovered from the reaction mixture was also found to exhibit the ability to promote NADPH-dependent reduction of exogenous DPIP.

NADPH oxidation in the presence of exogenous DPIP (Fig. 2). The DPIP–rIucD complex (4 μM) effects approximately a threefold increase in the rate of NADPH oxidation relative to that noted with DPIP alone. The process appears to involve an exchange of reducing equivalents between the protein-bound DPIP and that free in the medium. Further studies of the above-noted phenomenon of reducing equivalent exchange have revealed the process to require unmodified DPIP, since its conjugate with 2-mercaptoethanol fails to elicit NADPH oxidation by rIucD. Furthermore, rIucD preparations that do not show the presence of DTNB-titrable thiol groups and are devoid of lysine:N^6-hydroxylase activity (a situation that develops on prolonged storage of the protein) have been found to catalyze NADPH oxidation in the presence of DPIP. Similarly, rIucD preparations with thiol functions modified by either DTNB or NTCB still retain their ability to promote DPIP-dependent NADPH oxidation. Hence, it would appear that a noncovalent interaction between rIucD and DPIP could result in the formation of a species that is also capable of promoting NADPH oxidation. It has not been possible, however, to demonstrate the formation of such a noncovalent complex between the protein and the dye presumably due to its facile dissociation even under mild conditions em-