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Studies on the histidine residues in pigeonpea (*Cajanus cajan* L.) urease

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Abstract

The pH dependence of pigeonpea urease catalysis reveals the presence of two ionizable groups of pK_a values of 6.2 ± 0.1 and 8.8 ± 0.1, respectively. When urease was treated with excess diethylpyrocarbonate (DEP) at pH 6.8, a time-dependent exponential decay of its activity was observed and the pseudo-first order rate constant was proportional to the reagent concentration. The loss of activity was accompanied by a parallel increase in absorbance at 242 nm. Titration of urease with DEP revealed the presence of 12.9 ± 0.1 'accessible' histidine groups per hexameric pigeonpea urease. Hydroxylamine did not provide significant recovery of the DEP inactivated enzyme. Spectroscopic studies, circular dichroism and fluorescence show no effect of DEP on the gross conformation of urease. Irradiation of urease with visible light in the presence of small concentrations of the basic dyes like Rose Bengal or Methylene Blue at pH 6.8, brought about a time-dependent first-order decay of enzyme activity. Urea and acetohydroxamate (AHA) protect the enzyme against the inactivation by DEP or photo-oxidation. The inactivation reaction with the DEP or Rose Bengal was found to be linearly related to the blocking of 12 essential histidine groups per hexamer for complete inactivation. Since, each protein molecule is known to possess two catalytic units per hexamer hence, we propose that urease possesses at least one essential histidine per catalytic unit. The significance of these results is discussed.

Keywords: Urease; Diethylpyrocarbonate; Histidine reactivity; Photo-oxidation; Chemical inactivation

1. Introduction

The enzymatic hydrolysis of urea by the Ni-containing enzyme urease (urea amidohydrolase EC 3.5.1.5) occurs at a rate 10^{14} times faster than the uncatalysed reaction, yielding ammonia and carbon dioxide [1,2]. Much pathology is associated with the activity of ureolytic bacteria, and the efficiency of soil nitrogen fertilisation with urea is severely decreased by microbial urease activity [3,4]. For unknown reasons, some seeds are particularly rich sources of urease, and the most extensively studied example of this enzyme comes from seeds of the jack bean (*Canavalia ensiformis*). Urease was originally isolated as a pure, crystalline enzyme by Sumner [5] and these crystals, played a decisive role in proving the proteinacious nature of enzymes. Urease has been reported to be present in all tissues of soybean [6] and recently, the genes involved in nickel insertion into urease and the one encoding Ni-binding protein necessary for urease activity have been identified [7,8].

The structure and mechanism of urease is best characterised for the enzymes derived from *Klebsiella aerogenes* [9] and *Bacillus pasteurii* [10]. The pH dependence of *K. aerogenes* urease

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shows presence of two ionizable groups of $pK_a \cong 6.55$ and 8.85, respectively. Chemical modification studies with the histidine-selective reagent diethylpyrocarbonate (DEP) were compatible with a histidine residue serving as the general base [11]. Later, through site-directed mutagenesis it has been shown that His^{320} is essential for activity and catalysis [12].

Sakaguchi et al. [13] have shown the presence of histidine groups in jack bean urease through photo-oxidation studies. Barth and Michel [14] suggested the presence of histidine residues concerned in the catalytic activity of jack bean urease based on kinetic analysis and inhibition studies using a histidine reagent L-1-tosylamide-2-phenyl ethyl chloroethyl ketone.

Urease from dehusked pigeonpea (*Cajanus cajan* L.) seeds has been purified to homogeneity and partially characterised [15,16] and shown to be an important enzyme for potential clinical applications [17–20]. We have recently reported the correlation of thiol groups and activity of pigeonpea urease [21]. However, compared to the detailed inactivation studies available from *K. aerogenes* for essential histidine, using group specific reagent, DEP [11,22], no corresponding reports on histidine group studies have been made available on plant urease. Therefore, in the present communication an effort has been made to provide evidence for the involvement of histidine residues in the catalytic activity of urease from pigeonpea. Experiments have been described which throw light on their relative location in the active site vis-a-vis that of the SH groups. Furthermore, physico-chemical characterisation using CD and fluorescence studies have been used to rule out any gross conformational changes on urease by DEP.

2. Materials and methods

2.1. Materials

DTNB (5,5′-dithiobis-(2-nitrobenzoate), DEP, acetohydroxamate (AHA), Bovine serum albumin (BSA) and triethanolamine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rose Bengal, Methylene Blue and hydroxylamine were from CDH, India. Tris, urea (enzyme grade), trichloroacetic acid (TCA), Na_2HPO_4/NaH_2PO_4 were from Sisco Research Labs (Mumbai, India). Nessler's reagent was procured from Hi-Media Labs (Mumbai, India). All other chemicals were of analytical grade. All solutions were prepared in triple distilled water from an all-quartz distillation assembly.

2.2. Enzyme

Urease was purified from dehusked pigeonpea (*Cajanus cajan* L.) seeds, as described earlier [15]. The isolated enzyme was more than 95% pure as judged by native and SDS-PAGE. The specific activity of the purified enzyme varied from batch-to-batch in the range of 4500–5500 units mg^{-1} protein.

2.3. Enzyme and protein assay

For routine assay of urease activity, ammonia liberated in a fixed time interval at an enzyme saturating concentration of urea was determined by using Nessler's reagent [19]. The yellow colour produced was measured spectrophotometrically at 405 nm. The amount of ammonia liberated in the test solution was calculated by calibrating the reagent with standard ammonium chloride solution. An enzyme unit has been defined as the amount of enzyme required to liberate 1 μmole of product ammonia per minute under our test conditions (0.1 M urea, 0.05 M Tris–acetate buffer, pH 7.3, 37°C). Protein was assayed by the method of Lowry et al. [23] with BSA as standard.

2.4. Determination of pK_a values

The effects of pH on V_{max} and K_m was established by assaying urease activity in buffers containing 50 mM buffer, at the indicated pH values.

2.5. Inactivation of urease with DEP

DEP was dissolved in ethanol immediately before use. DEP concentration was measured by reacting an aliquot with 10 mM imidazole (pH 6.8) and monitoring the absorbance at 230 nm using an extinction coefficient of $3 \times 10^3\ M^{-1}\ cm^{-1}$ [24]. Desired volume of DEP, was added to the enzyme solution (1.41 mg protein ml^{-1}) in 60 mM sodium phosphate buffer, pH 6.8 at 30°C. DEP concentration was varied in the range

0.1–0.3 mM. The reaction was monitored at 242 nm ($\Delta\varepsilon$ 3.2×10^3 M^{-1} cm^{-1}) as described earlier [24,25] and for kinetic studies aliquots withdrawn at different time intervals from DEP-urease mixture as stated above, were assayed for residual activity using urea as substrate. Hydroxylamine reactivation studies of the DEP-inactivated enzyme were carried out by incubating it with 0.1 M hydroxylamine and time-dependent recovery of activity was monitored at 37°C.

In a separate set of experiments, the absorbance of enzyme–DEP reaction mixture at 242 nm and percentage residual activity was recorded in aliquots withdrawn at different time intervals in order to monitor the progress of modification of imidazole groups of the enzyme. Also, the residual SH groups were assayed for inactivated enzyme sample (residual activity 14%) by the method of Ellman [26].

For protection experiments, enzyme was incubated with DEP (0.1 mM) in the presence of urea (0.1 M) or AHA (0.1 mM). Aliquots withdrawn at different time intervals were assayed for the residual activity and increase in absorbance at 242 nm was also monitored. In each assay spontaneous hydrolysis of DEP was accounted and appropriate correction has been done.

2.6. CD and fluorescence studies

CD measurements were done on a JASCO 500A spectropolarimeter equipped with a 500N data processor. The instrument was calibrated with a 0.1% D-10-camphorsulfonic acid solution [27]. Conformational changes in secondary structure of protein urease were monitored between 220 and 270 nm with a protein concentration of 0.1 mg ml^{-1} in a 1-mm path length cuvette in absence and presence of 0.1 mM DEP. The baseline spectrum was subtracted from each spectrum. Each spectrum represents the average of three scans. The results are expressed as the mean residue molar ellipticity (θ) ($^\circ$ cm^2 $dmol^{-1}$), which is defined as $(\theta) = \theta_{obs} \times MRW/(10lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in g ml^{-1} and l is the length of the light path in centimeters. A mean residue weight (MRW) of 112 is used. Samples for all spectroscopic measurements were filtered through 0.45 μm membrane filters, and the exact concentration of the protein was determined by absorbance. All spectra were recorded at 27°C.

Fluorescence measurements were carried out on a Perkin-Elmer LS-50B spectrofluorimeter equipped with a constant temperature cell holder. The protein concentration was 0.05 mg ml^{-1}. For tryptophan and tyrosine fluorescence of the protein in absence and presence of 0.1 mM DEP, excitation at 292 and 280 nm, respectively, and the emission was recorded from 300 to 400 nm with 10 and 5 nm slit widths for excitation and emission.

2.7. Photo-inactivation of pigeonpea urease

The enzyme and dye (Rose Bengal or Methylene Blue) in 60 mM sodium phosphate buffer, pH 6.8 were irradiated in a water bath at 37°C in the presence or absence of substrate urea using a 100 W tungsten lamp kept at 18 cm from the sample. Aliquots withdrawn at different time intervals were assayed for the residual activity. The presence of small concentration of dye (~0.05 μg ml^{-1}) introduced into the activity assay solution with the enzyme aliquot had no effect on activity measurement.

In a separate set of experiment, Rose Bengal inactivated enzyme (residual activity 17%) was assayed for residual 'accessible' SH groups as described above.

3. Results

3.1. Determination of pK_a values

Urease retained full activity when assayed under standard conditions, following a 10 min incubation at 37°C in buffer pH range 5–10, however, the enzyme was rapidly inactivated outside this pH range.

The effect of pH on the kinetic constant for urease was determined in buffers at various pH values. The K_m for urea was partially constant over the pH range at which enzyme was assayed, i.e. K_m varies slightly from ca. 2.8 to 3.2 mM (data not shown). In contrast the V_{max} exhibited an optimum from pH 6.8–7.8. The effect of pH on $\log(V_{max})$ is shown in Fig. 1. The slope was 1 below the optimum pH and −1 above it, with inflection points at pH values of 6.2 ± 0.1 and 8.8 ± 0.1 [28]. These pK_a values were not due to the substrate urea, as its pK_a values do not lie in this range [9].

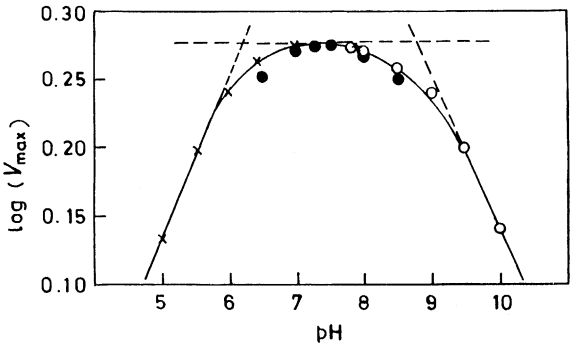

Fig. 1. The effect of pH on log(V_{max}) for pigeonpea urease. Urease V_{max} values were calculated and the log of the ratio is plotted here in arbitrary units versus the pH of the assay buffer. Buffers include Tris–acetate (○), HEPES-KOH (●) and citrate-phosphate (×).

3.2. Assay of histidine groups of urease with DEP

Freshly purified pigeonpea urease (2.47 mg ml^{-1}) was incubated in 60 mM phosphate buffer, pH 6.8, containing 500 μM DEP for 30 min. The change in absorbance was monitored versus a reference cuvette containing urease in buffer without DEP (Fig. 2). Modification of histidine residues with DEP leads to an increase in absorbance at 242 nm with an extinction coefficient of 3.2×10^3 M^{-1} cm^{-1}. Our spectroscopic results are consistent with modification of 12.9 ± 0.1 histidine residues per mole of native enzyme.

Assay of 'accessible' histidine groups of pigeonpea urease (1.41 mg ml^{-1}) was monitored with excess DEP (100 μM) in 60 mM phosphate buffer pH 6.8, 30°C and the increase in absorption with time was monitored at 242 nm (Fig. 3, (●)). If the correction is applied for very slow reacting phase to zero time by extrapolation, the absorbance increase corresponds to the reaction of 12.9 ± 0.1 'accessible' histidine groups per hexamer of enzyme protein molecule (molecular mass, 480 kDa), i.e. approximately two histidine groups per monomeric subunit, which is consistent with our spectroscopic data. The loss in percentage residual activity due to DEP inactivation is also shown in (Fig. 3, (○)).

3.3. Kinetics of DEP inactivation

A semi-log plot for the loss in residual enzyme activity due to modification of reactive histidine groups of pigeonpea urease has been shown in Fig. 4. Urease loses its activity in a single exponential decay when incubated with excess DEP at pH 6.8 and 30°C. Rate

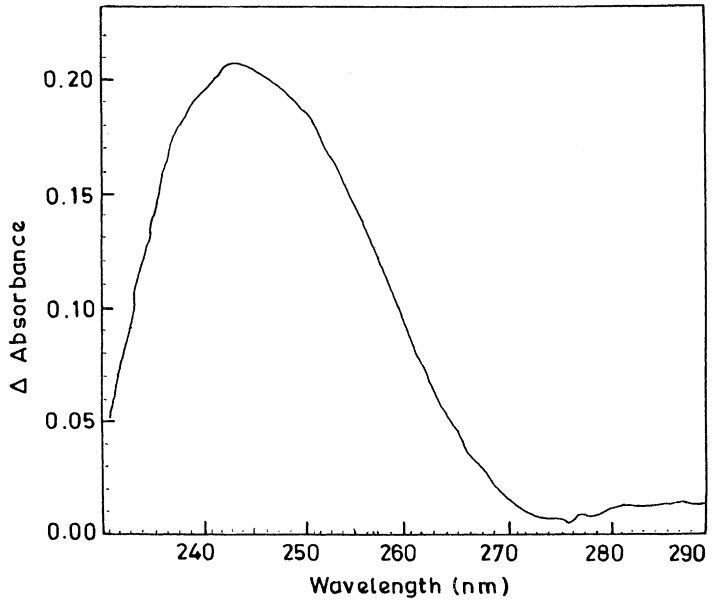

Fig. 2. Spectroscopic analysis of pigeonpea urease modification by DEP. Urease (2.47 mg ml^{-1}) was incubated for 30 min with DEP (0.5 mM) in 60 mM phosphate buffer, pH 6.8 at 30°C. The difference spectrum is shown for this sample minus untreated urease in same buffer.

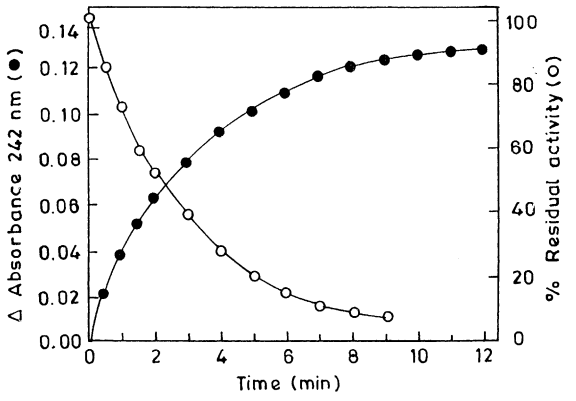

Fig. 3. A comparison of loss of catalytic activity and increase in absorbance at 242 nm on treatment of pigeonpea urease with DEP. Urease (1.41 mg ml^{-1}) and DEP (0.1 mM) were taken in 60 mM phosphate buffer (pH 6.8) at 30°C and absorbance increase at 242 nm was monitored at different time intervals (●). The loss in percentage residual activity is also shown (○).

constants are shown in Table 1. The pseudo first-order rate constants of the inactivation reactions were proportional to the DEP concentration. The values were 0.362, 0.690 and 0.995 min^{-1} at DEP concentrations equal to 0.1, 0.2 and 0.3 mM, respectively. A double-log plot (i.e. log(k_{app}) versus log(DEP)) is found to be linear with slope equal to 1.02 (Fig. 4 inset).

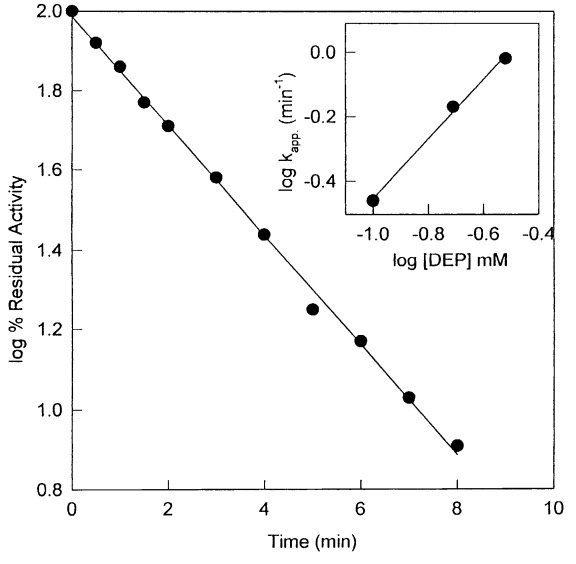

Fig. 4. Kinetics of inactivation of pigeonpea urease by DEP. The enzyme (1.41 mg ml^{-1}) was incubated with 0.1 mM DEP in 60 mM phosphate buffer (pH 6.8) at 30°C and aliquots withdrawn at different time intervals were assayed for residual activity. Inset, log(k_{app}) inactivation is plotted against log[DEP].

Table 1
Rate constants for inactivation reactions

Reagent concentration (mM)	k (min^{-1})
DEP[a] (0.1)	0.385 ± 0.024
DEP[b] (0.1)	0.372 ± 0.027
Rose Bengal[b] (2.5)	0.022 ± 0.002
Methylene Blue[b] (0.05)	0.041 ± 0.004

[a] Kinetics of reaction of histidine groups based on absorbance change at 242 nm.
[b] Kinetics of inactivation of the enzyme based on activity measurements at 405 nm.

The treatment of DEP inactivated enzyme with 0.1 M hydroxylamine led to only partial recovery of activity (15%) on 45 min of incubation (data not shown).

When enzyme was treated with DEP (0.1 mM) in presence of 0.1 M urea or 0.1 mM AHA, a significant protection in rate of inactivation was observed. Approximately 42 and 36% reduction in k was monitored with urea and AHA, respectively (Fig. 5). Although a portion of the urea was degraded under these experimental conditions, there was sufficient buffer capacity to prevent significant pH change and the urea concentration remained saturated during the short time required for the analysis.

3.4. Conformational studies

Changes in the secondary structure of pigeonpea urease, if any, upon treatment with DEP, were monitored by optical spectroscopy. Far-UV CD spectra of native and 0.1 mM DEP treated urease has been shown in Fig. 6. The molar ellipticity at 220 nm was $21 \times 10^{-3\circ}$ cm^2 dmol^{-1} for DEP treated urease, while that of the native urease is $20 \times 10^{-3\circ}$ cm^2 dmol^{-1}. This difference in molar residue ellipticity is insignificant, thus, it can be concluded that DEP treatment of pigeonpea urease does not lead to any gross conformational changes in the protein structure.

Fluorescence spectral studies of pigeonpea urease in presence and absence of 0.1 mM DEP are also similar, indicating no significant changes (data not shown). Thus, it can be concluded that DEP treatment does not bring any conformational changes to the structure of urease.

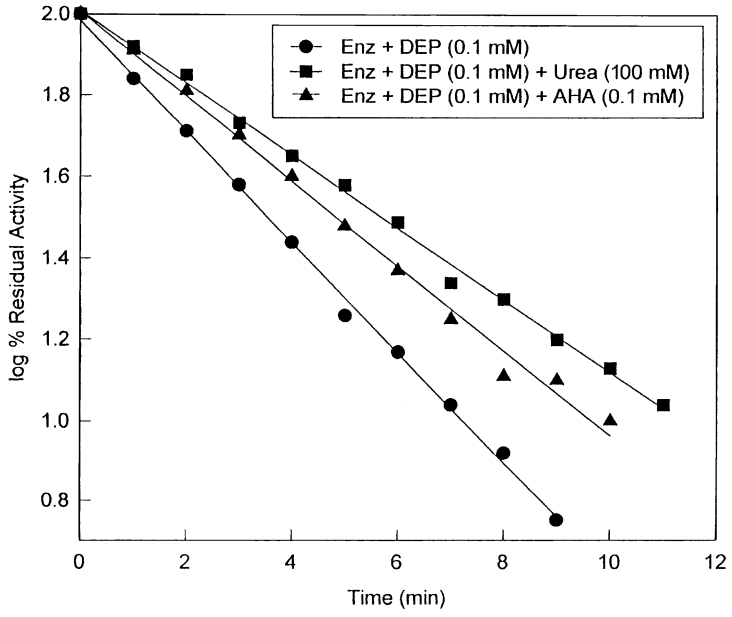

Fig. 5. Effect of substrate urea and AHA on the inactivation of pigeonpea urease by DEP. Enzyme (1.41 mg ml^{-1}) and DEP (0.1 mM) were incubated in the presence of urea (100 mM) or AHA (0.1 mM). Residual activity was assayed in aliquots withdrawn at different time intervals.

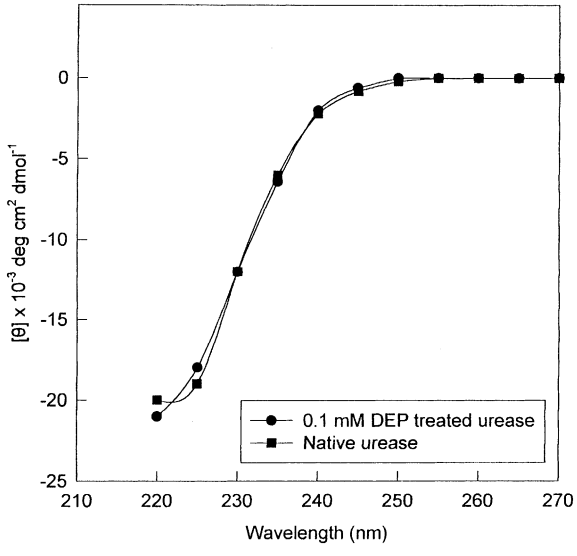

Fig. 6. Far-UV CD spectra of native (■) and DEP (0.1 mM) treated (●) pigeonpea urease. Spectra were recorded in 1 mm cells with a protein concentration of 0.1 mg ml^{-1} at 27°C. The baseline spectrum was subtracted from each spectrum.

3.5. Photo-inactivation of pigeonpea urease

When a solution of pigeonpea urease is irradiated with visible light at pH 6.8 and 37°C in the presence of 2.5 mM Rose Bengal, the enzyme loses its catalytic activity fairly rapidly in an exponential decay (Fig. 7) with a rate constant $0.022 \pm 0.002\,\text{min}^{-1}$. Methylene Blue (0.05 mM) showed similar pattern of inactivation (data not shown) with a rate constant of $0.041 \pm 0.004\,\text{min}^{-1}$ (Table 1). Enzyme solutions irradiated in the absence of dye or kept in the dark in presence or absence of dye did not show any appreciable loss of activity over the same period of time. Rate of photo-inactivation decreases sharply as pH is decreased (data not shown). Furthermore, substrate urea protects the enzyme significantly against the photochemical inactivation (Fig. 7). The rate constant of photochemical inactivation showed a 40% decrease in the presence of 0.1 M urea, against the Rose Bengal inactivation.

In a separate set of experiment, the DEP inactivated enzyme (residual activity 14%) and Rose Bengal inactivated enzyme (residual activity 17%)

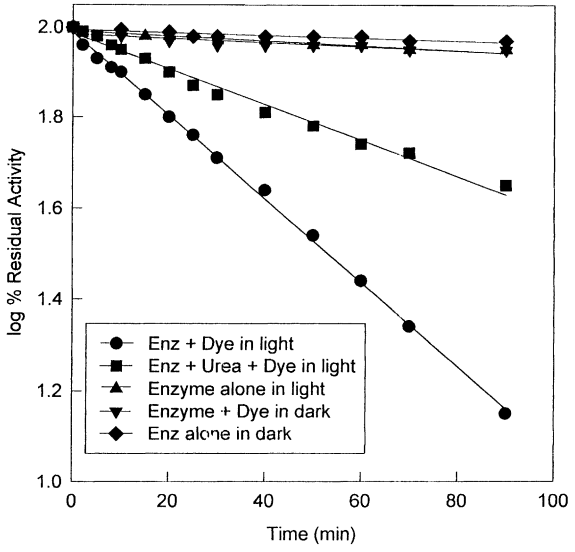

Fig. 7. Inactivation of pigeonpea urease on irradiation in the presence of Rose Bengal (2.5 mM) in the absence or presence of urea (100 mM) as described in materials and methods at 37°C and residual activity in aliquots withdrawn at different time intervals were determined.

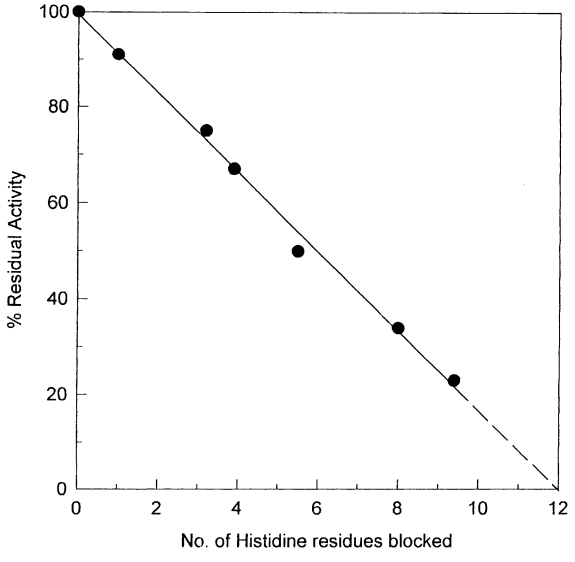

Fig. 8. Relationship between modification of histidine residues and inactivation of pigeonpea urease on treatment with DEP. The number of histidine residues modified were calculated from the absorbance increase at 242 nm as described in materials and methods.

were assayed for free SH groups. Rose Bengal was removed by dialysing the inactivated enzyme against 60 mM phosphate buffer for 2 h at 4°C. A total of 5.63 ± 0.1 and 5.43 ± 0.1 free SH groups per mole of the enzyme were titrated for Rose Bengal and DEP inactivated enzymes, respectively. Also, the kinetics of the inactivated urease with DTNB was identical to those described earlier for the native enzyme [21].

The number of histidine groups modified by DEP or Rose Bengal has been compared with the loss of catalytic activity at corresponding times. Representative data for DEP has been shown in Fig. 8. Extrapolation of the straight-line shows that complete inactivation may be achieved on modifying 12 histidine residues per molecule of the enzyme or two histidine residues per monomer.

4. Discussion

pH studies with pigeonpea urease using its physiological substrate urea on enzyme catalysed reaction, suggested that two acid–base dissociable groups are involved in the catalytic process. Their pK_a values were found to be 6.2 ± 0.1 (required in its basic form) and 8.8 ± 0.1 (required in its acidic form). Although such pK_a values are greatly perturbed, these values suggest tentatively the participation of an imidazole and SH group in the reaction catalysed by this enzyme, respectively. Presence of thiol groups in the activity of pigeonpea urease has been already shown recently [21]. Todd and Hausinger [29] have shown similar pH dependence of *K. aerogenes* urease catalysis with the presence of two ionizable groups; one group (p$K_a \cong 6.55$) must be deprotonated and second group (p$K_a \cong 8.85$) must be protonated for catalysis. Laidler [30] had given similar report for jack bean urease. Accordingly, the possibility of the involvement of an imidazole group in catalysis by pigeonpea urease has been followed up more carefully.

DEP reacts readily with imidazole groups and as such it is known to modify histidine residues of several enzymes, including urease from different sources at neutral pH [11,22,31,32]. Reaction of DEP with imidazole is known to be accompanied by an increase in absorbance approximately at 242 nm, due to the formation of an inactive ethoxyformylated enzyme derivative. The same was found to be the case

in the reaction of DEP with pigeonpea urease. From our spectroscopic studies (Fig. 2) and time-dependent absorbance increase at 242 nm (Fig. 3), the number of accessible histidine groups has been determined and found to be 12.9 ± 0.1 per mole of enzyme, i.e. approximately two groups per monomer of the hexameric pigeonpea urease. The microbial urease from *K. aerogenes* was also found to contain 12 moles of histidine/molecule of native enzyme through DEP derivatization studies [11]. Takishima et al. [33] have shown that out of 25 histidine residues in jack bean urease 13 were crowded in the region between residues 479 and 607, suggesting that this region may contain the nickel-binding site. Cys^{592}, which is required for activity, is located in the above-mentioned histidine-rich region. Preliminary X-ray absorption spectroscopy studies were interpreted for jack bean urease as indicating histidinyl ligation of the active site Ni [34]. However, more detailed X-ray absorption spectroscopic studies could only demonstrate that the legends were a mixture of nitrogen and oxygen atoms [35].

The number of histidine groups modified by DEP at different time intervals has been computed. The loss of activity was found to be linearly related with the number of histidine residues blocked and data extrapolated to complete inactivation of the enzyme on the reaction of 12 histidine residues per hexameric molecule of the pigeonpea urease (Fig. 8). Irrespective of the total number of histidine groups modified per subunit the present data strongly suggests that only one of these is catalytically important. For example, the rate of inactivation shows a first-order dependence on DEP concentration (Fig. 4, Table 1); higher order dependence should have been observed if more than the one-histidine residues are required for catalysis. In addition, the slope of the double-log plot is close to unity. Park and Hausinger [11] have also shown that urease from *K. aerogenes* possesses at least one essential histidine per catalytic unit.

DEP is also known to react with free SH groups, tyrosyl and lysyl side chains. Assay of residual free SH groups in DEP or Rose Bengal inactivated pigeonpea urease showed that about 92% of the readily accessible SH groups were still intact when the enzyme was completely inactivated. In the present case, the reaction with tyrosyl side chains is ruled out because of lack of any absorbance change at 278 nm [36]. On the other hand, reaction of DEP with lysyl residues is also ruled out, because that DEP is less likely to react with lysyl residues at below pH 7.0 because the protonated nucleophile does not react with this reagent. Under our experimental pH conditions (i.e. at pH 6.8) the reaction of DEP with protein is known to be specific for histidine residues [24].

In model compounds and with some proteins, the reaction of DEP with imidazole group is reversed on prolonged treatment with hydroxylamine. Treatment of DEP-inactivated pigeonpea urease with hydroxylamine led to only 15% recovery of the active enzyme. In contrast, full activity was restored when the *K. aerogenes* inactivated urease was treated with hydroxylamine. However, observations similar to ours have been made with several other ethoxyformylated enzymes [24,31,37–41]. According to Avaeva and Krasnova [40] and Miles [24], in such case NH_2OH treatment on the ethoxyformylated histidine leads to opening of imidazole ring by Bamberger reaction.

Miles [24] has advised physico-chemical studies of modified protein to rule out possible polymerisation or conformational changes, if the DEP has been used at sufficiently high concentrations. Our spectroscopic studies, CD and fluorescence clearly showed that there is no gross conformation change in the urease structure in the presence of 0.1 mM DEP.

Pigeonpea urease is rapidly inactivated on irradiation with visible light in the presence of a very small concentration of anionic dyes like, Rose Bengal or Methylene Blue, at neutral pH. Under these conditions, the method is reported to be specific for attack on imidazole groups [42,43]. This is supported by the observation that the rate of photo-inactivation decreases sharply as pH is decreased (data not shown) which is consistent with the report that protonated imidazole groups are resistant to photo-oxidation [42,43]. Sakaguchi et al. [13] have also shown the presence of histidine groups in jack bean urease through photo-oxidation studies using Methylene Blue and found to be necessary for both the binding ability and the enzymatic activity of the enzyme.

Urea (0.1 M) protects the enzyme significantly, but not very strongly towards the DEP and dye inactivation (Figs. 4 and 5). Similar protection pattern was also obtained with AHA. AHA is a known competitive inhibitor of urease from pigeonpea [44], jack bean [45] and bacterial urease [46,47]. The present

data suggests that protection of the enzyme against the DEP or photo-inactivation is primarily due to the binding of urea and AHA to the same catalytic sites. Thus, both DEP and photo-inactivation studies strongly suggest that pigeonpea urease active site has at least one essential histidine residues per monomer of the hexameric urease.

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