CHAPTER V
PRESENCE OF ESSENTIAL HISTIDINE RESIDUES AT THE ACTIVE SITE OF
A. fischeri NITRITE REDUCTASE
A. fischeri nitrite reductase was inactivated by the histidine-specific reagent, diethylpyrocarbonate (DEPC), at pH 6.8 and 6.5 at 4°C. The rate of inactivation was faster at pH 6.8 as compared to that at pH 6.5. The inactivation was found to be concentration- and time-dependent. The inactivation rates followed pseudo-first order kinetics. On treatment of the enzyme with DEPC the absorbance around 240 nm increased which is specific for N-carbethoxyimidazole derivative. The absorbance remained unchanged around 280 nm region suggesting that tyrosine residues were not modified. Free sulfhydryl groups were not modified during the inactivation of the enzyme with DEPC. Though four histidine residues have been shown to be modified for the complete inactivation of the enzyme with DEPC, only one histidine residue per molecule of the enzyme has been shown to be essential for loss of activity on the basis of the reaction order \((n)\) which was calculated to be 0.9. The inactivated enzyme was reactivated by 0.75 M hydroxylamine at neutral pH which caused removal of ethoxyformyl groups from the modified enzyme. The substrate of the enzyme, nitrite, considerably protected the enzyme activity against inactivation by DEPC. The maximum protection by nitrite was obtained at a concentration of 5 mM or above. The addition of nitrite at concentrations less than 0.2 mM showed no protection against DEPC inactivation. The pKs of the ionizable groups of the enzyme which are required for the activity of the enzyme were 7.0 and 7.7. These correspond to the imidazole group. These studies suggested that one histidine residue is essential for the activity of the enzyme.
INTRODUCTION

Nitrite reductase from *A. fischeri* catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). The enzyme has a relative molecular mass of 80,000 and is composed of two subunits of equal size which are covalently bonded by a disulfide bridge. Methionine has been found as the sole N-terminal residue (Husain and Sadana, 1974a; 1974b). It contains two c-type hemes per molecule but no nonheme iron or molybdenum. It is, therefore, distinct from assimilatory nitrite reductases and sulfite reductases, which contain siroheme and iron-sulfur centres (Lancaster *et al.*, 1979; Siegel *et al.*, 1982; Krueger and Siegel, 1982) and for which electron donor is ferredoxin. Recently Liu and Peck (1981) have reported a new type of nitrite reductase from a strain of *D. desulfuricans* which contains six c-type hemes per molecule and resembles the nitrite reductase from *A. fischeri* in having c-type hemes.

Reduction of hydroxylamine is also catalyzed by *A. fischeri* nitrite reductase. It has been reported that both the substrates, nitrite and hydroxylamine, are reduced at the same active site and no free intermediate has been detected during the six-electron reduction of nitrite to ammonia (Prakash and Sadaña, 1972). Similar data have been reported by Cresswell *et al.* (1965); Hageman *et al.* (1962); Lazzarini and Atkinson (1961); Ramirez *et al.* (1966).

Very little information is available on the amino acid residues which are involved at the catalytic site of nitrite reductase. *A. fischeri* nitrite reductase was not affected by *p*-HMB (4 - 5 moles/mole enzyme) or DTNB (50 moles/mole enzyme),
specific reagents for sulfhydryl groups (Husain and Sadana, 1974a). However, in the presence of large excess (400 - 800 fold) of mercurial reagents, p-HMB and p-CMS, the enzyme activity was completely inhibited (Prakash and Sadana, 1972). This was interpreted that inhibition by -SH reagents was not due to mercaptide formation but involved some other interactions.

A mechanism of nitrite reduction which involves a hydroxyl group of an active serine as the substrate binding site, and the existence of bound intermediates of nitrite reduction was proposed by Kemp et al. (1963). Although the enzyme purified from E. coli by Kemp et al. (1963) would reduce NO\textsuperscript{-2}, NH\textsubscript{2}OH and SO\textsubscript{3}\textsuperscript{-2}, its physiological role was that of a sulfite reductase. The models proposed for NO\textsuperscript{-2} and SO\textsubscript{3}\textsuperscript{-2} reduction were similar. The Achromobacter nitrite reductase, however, did not reduce sulfite (Prakash and Sadana, 1972).

The work of Cresswell et al. (1965) showed that CN\textsuperscript{-} and p-HMB were inhibitory to nitrite reductase isolated from higher plants. They proposed that hydroxyl of a hemiacetal group could be the binding site for nitrite. The CN\textsuperscript{-} inhibition was presumed to be competitive and due to the reaction of cyanide with an active carbonyl group to form a cyanohydrin as previously proposed by Mager (1960). However, the lack of inhibition of nitrite reductase from higher plants by phenyl methyl sulfonyl fluoride (PMSF) or fluoride (Dalling et al., 1973), hydroxyl modifying agents, or phenyl hydrazine (Dalling, 1972), a carbonyl modifier, does not support the models proposed by Cresswell et al. (1965) and Hewitt et al. (1968). A histidine residue on the corn nitrite reductase was proposed by Loussart
and Hageman (1974), which reacts with nitrite to form nitrosamine.

Histidine as the binding site for nitrite was suggested because of the well known reaction of nitrite with secondary amines that produces relatively stable nitrosamine, and from the pH profile of the nitrite reductase (Hucklesby et al., 1972). There are many reagents known which can modify the histidine residues at the active site of an enzyme and cause inactivation. These include diethylpyrocarbonate (DEPC) (Bosshard, 1984; Gomi and Fujioka, 1983), diazo-1 H-tetrazole (Cohen, 1968) and bromoacetone (Beeley and Neurath, 1968). Whether a histidine residue is involved in the catalytic action of _A. fischeri_ nitrite reductase has been determined by chemical modification of the enzyme using DEPC as the histidine-specific reagent and studying the effect of pH on its kinetic parameters, _K_m_ and _V_max_ according to Dixon and Webb (1964).

DEPC has been shown to be specific for histidine residues below pH 7.0 from model studies (Muhlard et al., 1967). It reacts stoichiometrically with histidyl residues (Leskovac and Pavkov-Pericin, 1975; Wallis and Holbrook, 1973). The formation of N-carbethoxy-histidyl derivative is followed spectrophotometrically by the increase in absorbance between 230 - 250 nm (Ovadi et al., 1967; Ovadi and Keleti, 1969). Hydroxylamine removes the carbethoxy groups from N-carbethoxy-histidyl residues (Melchior and Fahrney, 1970). Inactivation of an enzyme by DEPC has been correlated with the modification of histidyl residues at the active site if hydroxylamine reactivates the enzyme (Ovadi and Keleti, 1969; Thome-Beau et al., 1971;
Setlow and Mansour, 1970; Horiike et al., 1979). However, Melchior and Fahrney (1970) have found that the reagent can also react with other nucleophilic residues which occur in proteins.

**METHODS**

Treatment of *A. fischeri* nitrite reductase with diethylpyrocarbonate (DEPC)

The carbethoxylolation of *A. fischeri* nitrite reductase was carried out at 4°C in 50 mM potassium phosphate buffer, pH 6.8.

As diethylpyrocarbonate (DEPC) gets hydrolysed rapidly, its concentration was determined just before its use as described below:

An aliquot of diluted stock solution of DEPC was added to 3 ml of 10 mM imidazole solution at pH 7.5 in a cuvette and increase in absorbance at 230 nm due to the formation of N-carbethoxyimidazole was determined. The concentration of DEPC was calculated from \( \Delta A = 3 \times 10^3 \text{ cm}^{-1} \text{M}^{-1} \) (Melchior and Fahrney, 1970).

For studying the effect of DEPC concentration on the inactivation of the enzyme, 0.1 mg of the enzyme was treated with various concentrations of DEPC (1, 2, 3, 5 mM). The enzyme activity of the DEPC-treated enzyme was assayed after different time intervals by drawing an aliquot from the reaction mixture. The extent of inhibition was calculated by comparing the activity of the modified enzyme to the reference enzyme treated in a similar manner but without DEPC. The diethylpyrocarbonate
was used as a solution in cold dry ethanol. The final concentration of ethanol in the reaction mixture was never more than 2%.

For studying the effect of pH on the enzyme inhibition by DEPC, 0.1 mg enzyme in 50 mM potassium phosphate buffer was treated with various concentrations of DEPC (1, 2, 3, 5 mM) at two different pH values of 6.5 and 6.8. The activity of the enzyme was assayed after different time intervals in reaction systems and controls as well.

The apparent first order rate constant of inactivation depends on the concentration of the modifier and can be expressed by the following equation:

\[ K_{app} = K(M)^n \]

where \( K_{app} \) is the apparent first order rate constant for the inactivation, \( K \) is the second order rate constant, \( M \) signifies the concentration of the modifier and \( n \) is a number equal to the average order of the reaction with respect to the concentration of the modifier. Taking the logarithm of both sides:

\[ \log K_{app} = \log K + n \log (M) \]

\( K_{app} \) can be calculated from a semi logarithm plot of the residual enzyme activity as a function of time. The order of the reaction \( (n) \) can be experimentally estimated by determining \( K_{app} \) at a number of different concentrations of the modifier. A plot of \( \log K_{app} \) against \( \log (M) \) should give a straight line with a slope equal to \( n \), where \( n \) is the number of molecules of modifier reacting with each active unit of the enzyme to produce
an enzyme-inhibitor complex (Levy et al., 1963; Ramakrishna and Benzamin, 1981; Marcus et al., 1976).

**Spectrophotometric study of A. fischeri nitrite reductase inhibition by DEPC**

Nitrite reductase (1 mg/ml) in 50 mM potassium phosphate buffer, pH 6.8 was taken in each of the two cuvettes, in the reference and sample compartments of a Gilford 250 spectrophotometer which was cooled with circulating water at 4°C. A solution of DEPC (0.01 ml/ml of the enzyme solution) was added to the sample cell to give a final concentration of 3 mM and the same amount of ethanol was added to the reference cell. Difference spectra were recorded at different time intervals (5, 10, 15, 20, 30, 45, 60 min) between 300 nm and 235 nm. The number of histidyl residues modified by DEPC was determined using $\Delta \varepsilon = 3200 \text{ cm}^{-1} \text{M}^{-1}$ at 240 nm (Ovadi et al., 1967). An aliquot (0.01 ml) of the reaction mixture was removed from both the cuvettes after the absorbance at 235 nm was recorded, and assayed for enzyme activity. The time referred to was the time when the spectrum was completed and an aliquot was removed for the assay.

**Effect of substrate on inhibition during treatment of A. fischeri nitrite reductase with DEPC**

The enzyme (0.1 mg) in 50 mM potassium phosphate buffer, pH 6.8, was preincubated with different concentrations of nitrite (1.8 - 18 mM) for one min at 4°C, before the addition of DEPC (3.0 mM). The control samples were also treated under identical conditions but without nitrite. The aliquots were
withdrawn from both the treated enzyme and the controls at various time intervals and checked for nitrite reductase activity.

**Effect of hydroxylamine on reactivation of DEPC - inactivated A. fischeri nitrite reductase**

Initially the enzyme was treated with 3 mM DEPC. Aliquots of the treated enzyme were withdrawn at different time intervals and incubated in 100 mM potassium phosphate buffer, pH 7.0, containing 0.75 M hydroxylamine hydrochloride (adjusted to pH 7.0 with 0.1 N KOH) at 4°C. The enzyme activity was estimated after 45 min of incubation.

**Estimation of free sulfhydryl groups**

Free sulfhydryl groups of nitrite reductase, before and after treatment with DEPC, were estimated by titration of the enzyme in the presence of 8 M urea with p-HMB as described by Benesch and Benesch (1962). The solution of p-HMB (sodium salt) was prepared by dissolving 8 mg of the compound in 1 ml of 0.04 N NaOH and diluted to 25 ml. The p-HMB solution was standardized both spectrophotometrically by recording the absorbance at 232 nm ($\varepsilon_m = 1.69 \times 10^4$) (Boyer, 1954) and by titration against standard reduced glutathione solution as described by Benesch and Benesch (1962). The determinations by the two methods were in good agreement.

The *A. fischeri* nitrite reductase samples were incubated with 8 M urea for 60 min. An accurately measured aliquot of the enzyme in 50 mM potassium phosphate buffer, pH 7.0 was placed in one ml stoppered silica cuvettes. Small aliquots (10 μl) of standard p-HMB solution were added to the experimental solution and the blank which contained equal volume of the buffer
The contents of the cuvettes were mixed and absorbance recorded at 250 nm after each addition. The \( \text{pHMB} \) solution was added till there was no change in the absorbance. The observed absorbances were corrected for dilution and plotted against the volume of the \( \text{pHMB} \) added. The end point is obtained from intersection of the two lines.

**Kinetics**

The kinetic parameters, \( K_m \) and \( V_{max} \) of *A. fischeri* nitrite reductase, were determined at different pH values by Lineweaver-Burk method. The final concentration of nitrite in the reaction mixtures varied from 18 mM to 260 mM. The potassium phosphate buffer was used in the pH range 5.5 - 8.5. For each experiment the pH was determined after completion of the reaction. The kinetic parameters, \( pK_m \) (\( -\log K_m \)), \( \log V_{max} \) and \( \log V_{max}/K_m \), were plotted against pH for the determination of \( pK_m \) values of ionizable groups according to the procedure of Dixon and Webb (1964).

**RESULTS**

**Inactivation of nitrite reductase with DEPC**

During preliminary experiments for the inactivation of nitrite reductase by DEPC, it was observed that nitrite reductase activity was inhibited by low concentration of DEPC (0.2 mM), but the rate of inactivation was very slow. In order to determine the appropriate concentration of the modifying reagent (DEPC) for the inactivation studies, various concentrations of the reagent were tested for the inactivation of nitrite reductase. Inactivation of nitrite reductase as a function of
Diethylpyrocarbonate concentration (Fig. 10) shows that the enzyme is more or less completely inhibited at 5 mM concentration of the modifying reagent (DEPC) at pH 6.8. The log of percent initial activity versus DEPC concentration is a linear function of the amount of the inhibitor (Fig. 10 inset). Plots of the log of percent initial activity with different concentrations of DEPC against time were linear up to nearly 10% of the initial activity (Fig. 11). This indicated that the inactivation process followed pseudo-first order kinetics with respect to time at any fixed DEPC concentration under these conditions.

Since nucleophiles are reactive in their unprotonated forms, Ovadi et al. (1967) proposed that DEPC should be selective for histidyl residues in proteins at pH 6.0. He showed that this was the case for several proteins. However, the reactivity of the nucleophiles in some proteins may not be the same as in model systems owing to different environments of the residues in these proteins. It is, therefore, considered advisable to determine the inhibition at more than one pH value and to consider the effect of pH on the stability and conformation of the enzyme being studied (Miles, 1977). The inactivation of nitrite reductase was carried out in the presence of different concentrations of DEPC at two different pH values, 6.8 and 6.5. It is evident from Table 6 that the rate of inactivation is at least two times faster at pH 6.8 compared to that at pH 6.5.

Spectrophotometric study of inactivation of nitrite reductase by DEPC

Diethylpyrocarbonate reacts with histidyl residues in
FIG. 10 Effect of DEPC concentration on inactivation of _A. fischeri_ nitrite reductase activity

- O——O 10 min
- □——□ 20 min
- △——△ 30 min
- ●——● 45 min

Insert shows the semilog plot of the same data.
FIG. 11 Inactivation of *A. fischeri* nitrite reductase by DEPC.

- o---o 1 mM DEPC
- □---□ 2 mM DEPC
- △---△ 3 mM DEPC
- ●---● 5 mM DEPC

Insert: Determination of the order of the reaction with respect to DEPC.
Table 6: Effect of pH on inactivation of *A. fischeri* nitrite reductase by DEPC

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*Note: The table is not fully visible in the image provided.*
model systems and in proteins to yield an N-carbethoxyimidazole derivative (Ehrenberg et al., 1976; Muhlard et al., 1967; Ovadi et al., 1967; Ovadi and Keleti, 1969). The reaction is conveniently followed spectrophotometrically by the increase in absorbance, which has a maximum between 230 and 250 nm (Ovadi et al., 1967; Ovadi and Keleti, 1969).

Spectrophotometric studies of the inactivation of nitrite reductase by DEPC were carried out at 4°C in a Gilford 250 spectrophotometer. Figure 12A shows a spectrum of the enzyme before and after treatment with 3 mM DEPC for 60 min (b). There was no change in the spectrum of the DEPC-treated enzyme above 270 nm indicating that tyrosine residues have not been modified, since O-carbethoxytyrosine absorbs between 270 and 280 nm (Muhlard et al., 1967). Figure 12A(a) shows the difference spectra of the enzyme at various time intervals (5, 10, 15, 20, 30, 45, 60 min) during the reaction of the enzyme with the modifying reagent. Large increases in absorbance at 240 nm, characteristic of N-carbethoxyhistidyl derivative, were observed. This is shown in Fig. 12A, 12B. It is evident from Fig. 12B that the plots of percent of initial activity against time, and difference absorbance at 240 nm against time, are mirror-image to each other. The relationship between percent initial activity and modified histidyl residues is shown in Fig. 12C. Extrapolation of the curve to 100% inhibition of the enzyme activity corresponds to carbethoxylation of 4 histidyl residues per molecule of enzyme.
FIG. 12A Effect of DEPC on the spectrum of *A. fischeri* nitrite reductase
(a) Difference spectra of the enzyme with 3 mM DEPC at 5, 10, 15, 20, 30, 45, and 60 min.
(b) Spectra of the enzyme before (—) and after treatment for one hour with 3 mM DEPC (——).
FIG. 12B  Effect of incubation time with 3 mM DEPC on the nitrite reductase activity (○—○) and difference absorbance at 240 nm (Δ—Δ).
FIG. 12C  Relationship between the number of histidyl residues modified and the nitrite reductase activity after treatment of the enzyme with 3 mM DEPC for various times.
MOLES HISTIDYL RESIDUES MODIFIED PER MOLE ENZYME

PERCENT OF INITIAL ACTIVITY
Effect of hydroxylamine on reactivation of DEPC-inactivated enzyme

Melchior and Fahrney (1970) found that hydroxylamine can remove N-carbethoxy group from N-carbethoxyimidazole derivative at pH 7.0 in several minutes. There are several reports where hydroxylamine has been used to reactivate the DEPC-inactivated enzymes (Ovadi and Keleti, 1969; Melchior and Fahrney, 1970; Setlow and Mansour, 1970; Thome-Beau et al., 1971; Huc et al., 1971; Horiike et al., 1979).

Figure 13 shows that *A. fischeri* nitrite reductase which has 20% or more initial activity can largely be reactivated by treatment with 0.75 M hydroxylamine at neutral pH in 45 min. The enzyme which has been inhibited 50% or less is almost completely reactivated, while the enzyme having less than 50% residual activity is not fully reactivated. For reactivation process, potassium phosphate and Tris-HCl buffers, were tried at the same pH values. Both the buffers gave similar results.

Effect of substrate on inactivation of nitrite reductase by DEPC

A range of nitrite (substrate) concentrations was used to determine if there was any protection in the inactivation of nitrite reductase by DEPC. Figure 14 illustrates that nitrite considerably protects the enzyme from inhibition by DEPC. The maximum protection by nitrite was obtained at a concentration of 5 mM or above; 2 mM nitrite provided some protection against inactivation. There was little or no protection of the inactivation process by 0.2 mM or less nitrite.
FIG. 13 Effect of hydroxylamine on the reversal of DEPC-inactivated *A. fischeri* nitrite reductase. (○—○) Enzyme treated with 3 mM DEPC. Aliquots of the enzyme, treated with DEPC were removed at various times and treated with hydroxylamine for 45 min (●—●).
FIG. 14 Effect of substrate (nitrite) on inactivation of A. fischeri nitrite reductase by DEPC.

•—• Enzyme treated with 3 mM DEPC in presence of 5 mM nitrite.

△—△ Enzyme treated with 3 mM DEPC in presence of 2 mM nitrite.

○—○ Enzyme treated with 3 mM DEPC in absence of nitrite.
Effect of DEPC on sulfhydryl groups modification

In order to determine if DEPC reacted with free sulfhydryl groups (Muhlard et al., 1967; Melchior and Fahrney, 1970), the total number of free sulfhydryl groups per mol of the enzyme was determined by spectrophotometric titration of the DEPC-treated and untreated enzyme with p-HMB (as described in Methods). Figure 15 represents results which are typical of several experiments performed in the presence of 8 M urea. It was observed that both untreated and treated enzyme having 20% of initial activity contained 3.8 sulfhydryl groups per mole of the enzyme.

Effect of pH on kinetic parameters

In order to obtain additional proof of the involvement of essential histidine residues for the activity of the enzyme, the dependence of the kinetic parameters, Michaelis Menten constant ($K_m$) and maximum velocity ($V_{max}$) of the nitrite reductase, on pH was studied. The pH dependence of $K_m$ and $V_{max}$ of the enzyme are shown in Fig. 16. According to Dixon and Webb (1964), the observed pH effects can be interpreted in terms of the pH values of the groups situated in the free enzyme, or in the free substrate, and in the enzyme substrate complex.

The $pK_m$-pH curve shows a group of two $pK$ values, 6.9 and 7.1 forming a wave. This could correspond to the same group of the enzyme, the ionization of which is affected by substrate binding so that its $pK$ is decreased in the enzyme substrate complex. Further examination of all the three curves shows that there are two ionizable groups showing $pK$ values around 7.0.
FIG. 15. Determination of sulfhydryl groups of DEPC-treated enzyme by spectrophotometric titration with p-HMB. The intercept, a, is due to absorption of the protein.
FIG. 16 Effect of pH on kinetic parameters $K_m$ and $V$ of *A. fischeri* nitrite reductase. Potassium phosphate buffer was used in the pH range 5.5 to 8.5. Kinetic parameters $pK_m$ ($-\log K_m$), $\log V$ and $\log V/K_m$ were plotted against pH according to the procedure of Dixon and Webb (1964).
and 7.7. The pK 7.0 corresponds to the intrinsic pK of imidazole group, and is in agreement with the assumption that a histidyl residue is essential for the activity of the enzyme, and is involved either near or at the active centre of the enzyme. Another pK value of 7.7 is also observed in all the three curves. It can be explained either as an imidazole group whose pK is different due to the environment or of a sulfhydryl group. But it has already been stated that free sulfhydryl groups are not required for activity of the enzyme and are also not modified by DEPC. Therefore, both the pK values of 7.0 and 7.7 are assumed for the ionization of imidazole groups.

**Number of histidyl residues essential for activity**

Under the conditions of Fig. 12C, about four histidyl residues per molecule of the enzyme were modified for the complete inactivation of the enzyme activity. This number of four histidyl residues was obtained by extrapolating the initial linear portion of the plot to zero activity. This method does not usually give the number of residues essential for activity (Tsou, 1962; Horiike and McCormick, 1979).

The number of essential histidyl residues or the residues present at the active site are calculated from the slope obtained from the plot of the logarithm of the apparent first order rate constant, $k_{app}$, versus the logarithm of the reagent (DEPC) concentration (Fig. 11 inset) (Ramakrishna and Benzamin, 1981). The slope of the curve (n) with respect to DEPC was determined to be 0.9. It suggests that loss of enzyme activity results from reaction of only one histidyl residue per molecule of the enzyme.
DISCUSSION

The inactivation of _A. fischeri_ nitrite reductase was studied using a histidine-specific reagent, diethylpyrocarbonate. This reagent was initially used by Rosen et al. (1966) to inactivate ribonuclease. Pradel and Kassab (1968) used the reagent to show the presence of histidine residues at the active site in creatine- and arginine kinases. Later on Ovadi and Keleti (1969) reported that DEPC reacts only with the histidine residues of glyceraldehyde-3-phosphate dehydrogenase. However, since that time, like many other group-specific reagents, it was shown to react with amino acid residues other than histidine. For instance, Melchior and Fahrney (1970) showed that DEPC can react with an active site serine of chymotrypsin and several amino groups of ribonuclease and only $\alpha$-amino group of pepsin at pH values as low as 4.0. In addition, it has been shown to react with histidine and tyrosine residues in thermolysine (Burnstein et al., 1974). Despite these limitations, the reagent has been widely used as a specific modifier of histidine at or near pH 6.0 (Ovadi et al., 1967).

When nitrite reductase was treated with DEPC, the enzyme activity was lost. The spectrophotometric studies were conducted to confirm whether inactivation of the enzyme was due to specific interaction of DEPC with histidine residues or not. The difference spectrum between the carbethoxylated and the native enzyme showed a characteristic positive peak at 240 nm which is characteristic of N-carbethoxyimidazole
The increase in absorbance at 240 nm is concomitant with the loss of enzyme activity. Inactivation of the enzyme activity is stoichiometric with modification of four histidyl residues per molecule of the enzyme. The specific modification of the enzyme at histidine residue is confirmed by the following observations: The reversal of the enzyme inhibition by 0.75 M hydroxylamine at pH 7.0 rules out the possibility that inactivation of the enzyme was due to the acylation of some of the amino groups of the enzyme (Melchior and Fahrney, 1970). The primary amines (α-amino and ε-amino of lysine) do not constitute a logical binding site for nitrite as the reaction leads to the production of nitrogen and an alcohol rather than a stable bound intermediate. The restoration of enzyme activity by hydroxylamine appears to rule out lysyl and arginyl residues since the more stable amide-like structures formed, when they are carbethoxylated, are not readily reversed by hydroxylamine (Miles, 1977; Muhlard et al., 1967; Melchior and Fahrney, 1970; Horiike et al., 1979). The lack of absorbance changes in 280 nm region during the treatment of the enzyme by DEPC shows that tyrosine residues are not modified by the reagent (Muhlard et al., 1967). It has also been generally believed that lysine and tyrosine side chains do not in any case react with diethylpyrocarbonate below pH 7.0 (Dykes and John, 1977).
Diethylpyrocarbonate is known to react with sulfhydryl residues in model systems (Osterman-Golkar et al., 1974; Berger, 1975). Recently, DEPC has also been shown to react with sulfhydryl groups of β-D-xylosidase from B. pumilus (Hilderson et al., 1984). The possible modification of sulfhydryl residues of nitrite reductase was tested by estimating free sulfhydryl groups in the DEPC-treated and native enzyme. The content of the free sulfhydryl groups of the native and DEPC-treated enzyme was found to be same indicating that -SH groups have not been modified by the reagent.

During the reversal of DEPC-inactivated enzyme by hydroxylamine it was observed that if the enzyme is inhibited for longer times, than the reactivation is not complete. The conformational changes have been implicated by Thome-Beau et al. (1971) in the incomplete regeneration of DEPC-treated arginine oxygenase. If there had been conformational changes in nitrite reductase, it would possibly prevent the access of hydroxylamine molecules to the carbethoxylated histidine residues and there would be incomplete regeneration of the ethoxy-formyl enzyme.

Several examples of the successful use of substrate for preventing or reducing the inhibitory effect of DEPC on enzymes have been cited in literature (Wallis and Holbrook, 1973; Sato and Uchida, 1975; Holbrook and Ingran, 1973; Burnstein et al., 1974) providing evidence that a histidyl residue is a catalytic group or is located at the substrate.
binding site. The inactivation of nitrite reductase by DEPC is prevented by 5 mM (or more) nitrite. This provides further evidence that a histidyl residue is the catalytic group or is located at the substrate binding site.

A study of the pH dependence of the kinetic parameters, $K_m$ and $V_{max}$, gave two main pK values, 7.0 and 7.7, and it is concluded that both the values correspond to imidazole group (Koshland, 1960). The difference in the two pK values may be explained on the basis of different environment of the two imidazole groups. Recently, Bosshard et al. (1984) have shown a histidyl residue with a pK = 8.0. Since $K_m$ varied with pH and was greater on both sides of the optimum pH, apparently the ionizable groups in the free enzyme control the binding of the substrate (Dixon and Webb, 1964).

Though four histidyl residues have been shown to be modified for the complete inactivation of the enzyme (calculated from the difference absorbance spectra), only one histidine residue is shown to be essential as shown by the inactivation reaction order (n). On the basis of kinetic data and the inactivation studies it has been concluded that the enzyme contains one catalytically essential histidyl residue.

Because of the limited stability of ethoxyformyl histidine residues (Holbrook and Ingram, 1973) it has not been possible to isolate the peptide containing the modified histidine residue and to identify the specific histidyl residues whose modification is responsible for loss of enzyme activity.