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An Essential Histidine Residue for the Activity of UDPglucose 4-Epimerase from *Kluyveromyces fragilis**

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UDPglucose 4-epimerase from *Kluyveromyces fragilis* was completely inactivated by diethylpyrocarbonate following pseudo-first order reaction kinetics. The pH profile of diethylpyrocarbonate inhibition and reversal of inhibition by hydroxylamine suggested specific modification of histidyl residues. Statistical analysis of the residual enzyme activity and the extent of modification indicated modification of 1 essential histidine residue to be responsible for loss in catalytic activity of yeast epimerase. No major structural change in the quarternary structure was observed in the modified enzyme as shown by the identical elution pattern on a calibrated Sephacryl 200 column and association of coenzyme NAD to the apoenzyme. Failure of the substrates to afford any protection against diethylpyrocarbonate inactivation indicated the absence of the essential histidyl residue at the substrate binding region of the active site. Unlike the case of native enzyme, sodium borohydride failed to reduce the pyridine moiety of the coenzyme in the diethylpyrocarbonate-modified enzyme. This indicated the presence of the essential histidyl residue in close proximity to the coenzyme binding region of the active site. The abolition of energy transfer phenomenon between the tryptophan and coenzyme fluorophore on complete inactivation by diethylpyrocarbonate without any loss of protein or coenzyme fluorescence are also added evidences in this direction.

UDPglucose 4-epimerase (EC 5.1.3.2) catalyzes a freely reversible reaction between UDPglucose and UDPgalactose. The mechanism of epimerization at C_4 carbon of the hexose moiety of the nucleotide sugar has been studied by several groups of workers (1-3). Highly purified enzymes from *Kluyveromyces fragilis* and *Escherichia coli* were used extensively for these mechanistic studies. Uridine nucleotide 4-ketosugar and NADH were unambiguously identified as enzyme-bound reaction intermediates. Subsequent hydride transfer from NADH and release of the reduced keto-intermediate lead to epimerization (2, 3). Mechanistically, the epimerase is, thus, regarded as a pyridine nucleotide-dependent oxido reductase where NAD acts as a true cofactor and not as a co-substrate. This epimerase has emerged as the prototype of a new class of oxido reductases that include among other such apparently dissimilar enzymes as dTDPglucose 4,6-dehydratase (EC 4.2.1.46), UDPglucuronate decarboxylase (EC 4.1.1.35), *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1), and 3-dehydroquinate synthase (EC 4.6.1.3). In all these cases, the oxidized substrate and the reduced coenzyme are initial transient intermediates on the enzyme surface. Gabriel and Van Lanten (4, 5) were the first to bring out the commonality of mechanism for such diverse enzymes. This has been documented further and critically reviewed recently by Frey (3). Apart from being a prototype of this class of oxido reductases, this epimerase has some other special features of interest that make it very relevant that the active site of the enzyme be mapped in detail.

This epimerase converts both the substrates, UDPglucose and UDPgalactose, to the common UDP-4-ketopyranose intermediate and hence is nonstereospecific with respect to glycosyl C-4 of the nucleotide sugar substrate (6). The stereospecificity of the pyridine nucleotide, on the other hand, is retained through the catalytic cycle (7). Further, the catalysis, as in the case of several dehydrogenases, is probably initiated by a general base that leads to the formation of enzyme-bound intermediates. For some of the classical dehydrogenases, a histidyl residue seems to function as the prototropic catalyst (8, 9). Earlier work in our laboratory on the reconstituted yeast enzyme system with Cibacron blue (10) and with etheno-NAD (11) indicated that significant tertiary structure homology at the pyridine nucleotide binding region of the active site exists between this epimerase and the dehydrogenases. In view of this emerging picture, it is important to explore whether the enzyme has any histidine residue critically placed in the neighborhood of the catalytic region of the active site. Earlier, Wong *et al.* (12, 13) had synthesized an active site-directed alkylating irreversible inhibitor of the enzyme by replacing the hexose moiety by a suitable phenyl derivative. This, however, failed to detect histidine or any other basic residue at the active site of the *E. coli* enzyme. In this report, employing diethylpyrocarbonate, a fairly specific modifying reagent for histidine residues for proteins under restrictive conditions (14, 15), we have identified the presence of an essential histidine residue in the active site of the yeast enzyme. Modification of this active histidine residue resulted in a coordinated loss of activity and a newly discovered protein energy transfer phenomenon through the coenzyme fluorophore. This modification also led to inaccessibility of the pyridine moiety of the coenzyme to reduction by borohydride. These results taken together suggest the histidine residue to be located in close proximity to the coenzyme fluorophore at the active site.

MATERIALS AND METHODS

Diethylpyrocarbonate was the product of Aldrich Chemical Co. Other biochemicals were purchased from Sigma. Highly purified and essentially homogeneous UDPglucose 4-epimerase was prepared by following up to stage III of the method of Darrow and Rodstrom explained in Ref. 4. Finally, the enzyme was passed through a Waters

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Protein Pak 300 SW HPLC column (7.8 × 300 mm) using 0.02 M sodium phosphate buffer, pH 6.7, as eluent. The specific activity of the enzyme was 40–60 units mg^{-1} of protein where 1 unit of enzyme could convert 1 μmol of UDPgalactose to UDPglucose min^{-1}. The enzyme activity was assayed spectrophotometrically according to the coupled assay procedure of Darrow and Rodstrom (4) or by a two-step assay method described previously (16). Protein was estimated by the method of Lowry *et al.* (17) or by measuring absorption at 280 nm and 260 nm. For stoichiometric calculations, the M_r for epimerase was assumed to be 125,000 as described earlier by Darrow and Rodstrom (4). Polyacrylamide gel electrophoresis was carried out according to the method of Davis (18). Fluorescence measurements were carried out with a Perkin-Elmer Cetus Instruments spectrofluorimeter model MPF 44B. Differential spectroscopy was carried out with a Cary 17 D spectrophotometer. Stock diethylpyrocarbonate was freshly diluted in absolute ethanol to prepare a working stock solution of required concentration. Stock concentration of diethylpyrocarbonate was determined spectrophotometrically by reaction with 20 mM L-histidine in 0.02 M potassium phosphate buffer, pH 7.0, and measuring the increase in absorbance at 230 nm ($\epsilon = 3000\ M^{-1}\ cm^{-1}$) (14). Carbethoxylation was carried out by incubating the enzyme with an appropriately diluted reagent in 0.02 M potassium phosphate buffer. The final concentration of ethanol in the reaction mixture ranged from 1% to 10% by volume and was found to have no significant effect on the activity and stability of the enzyme during the incubation time. The extent of inactivation was determined by measuring the residual enzyme activity on an aliquot removed from the reaction mixture. UDPglucose dehydrogenase, the coupling enzyme of the coupled assay procedure, was tested with diethylpyrocarbonate, but no inhibitory effect was observed at the concentrations used in the assay media. The amount of *N*-carbethoxyhistidine in epimerase in different stages of inactivation was calculated by difference spectra using a molar absorptivity of 3200 $M^{-1}\ cm^{-1}$ at 240 nm (14). The value of k' (first order rate constant for hydrolysis of diethylpyrocarbonate) was obtained by measuring the amount of diethylpyrocarbonate remaining at various times on treatment with 20 mM histidine in 0.02 M potassium phosphate buffer, pH 7.0, as described above.

RESULTS

Inactivation of UDPglucose 4-Epimerase with Diethylpyrocarbonate—Incubation of UDPglucose 4-epimerase with diethylpyrocarbonate in 0.02 M potassium phosphate buffer, pH 7.0, resulted in a time-dependent loss of enzyme activity. The activity remaining, correcting for decomposition of diethylpyrocarbonate in a buffered solution, is described by

$$\ln (A/A_0) = -k/k'\ I_0\ (1 - e^{-k't})$$

where the percent of activity remaining is A/A_0 at time t, I_0 is the initial concentration of diethylpyrocarbonate, k is the second order rate constant for reaction of the enzyme with the reagent, and k' is the pseudo-first order rate constant for degradation of the reagent (19). The value of k' for diethylpyrocarbonate was 63×10^{-3} min^{-1} and 28×10^{-3} min^{-1} in the presence of 1% and 10% ethanol, respectively. Since concentration of ethanol in the incubation mixture ranged from 1% to 10%, the kinetics of inactivation were followed in the presence of both 1% and 10% ethanol. Plots of log remaining activity against time $(1 - e^{-k't})/k'$ for diethylpyrocarbonate inactivation at various concentrations of the reagent yielded straight lines in both the cases. Fig. 1 represents the results of inactivation kinetics obtained with 10% ethanol. The plot of pseudo-first order rate constant values for inactivation (k_{obs}) against diethylpyrocarbonate concentration was linear, the slope yielding a second order rate constant (k) of 300 $M^{-1}\ min^{-1}$, respectively. The reaction order for inactivation with respect to diethylpyrocarbonate concentration was calculated from a plot of log k_{obs} against log reagent concentration (20). The reaction order obtained from the slope of the plot was approximately 1 (Fig. 1, *inset*) indicating that approximately 1 mol of diethylpyrocarbonate inactivated 1 mol of epimerase. A similar result was also obtained when diethylpyrocarbonate modification was carried out in the presence of 1% ethanol.

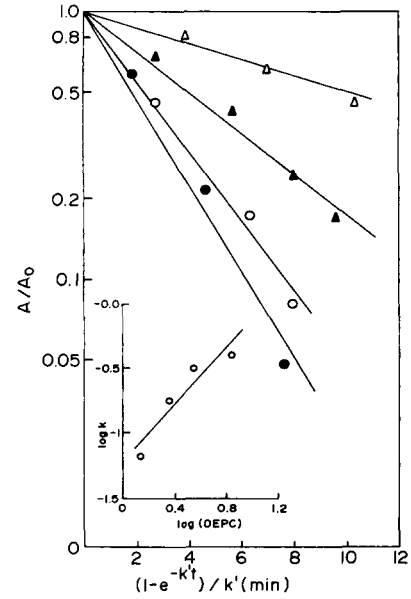

FIG. 1. **Kinetics of inactivation of epimerase by diethylpyrocarbonate.** The enzyme (0.125 mg/ml) was incubated with 0.6 mM (△), 0.68 mM (▲), 0.97 mM (○), and 1.36 mM (●) diethylpyrocarbonate in the presence of 10% alcohol in 0.02 M potassium phosphate buffer, pH 7.0, at 27 °C. At time intervals, aliquots were removed for measurement of the residual enzyme activity. Control enzyme having alcohol only was incubated under identical conditions in both cases. Control rates remained unchanged. *Inset,* plot of log (pseudo-first order rate constants for inactivation (k_{obs})) obtained at various concentrations of diethylpyrocarbonate against log concentration of the reagent.

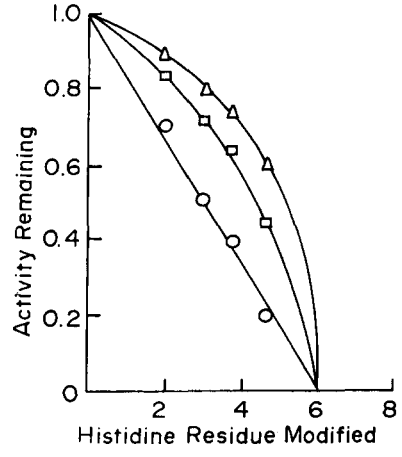

FIG. 2. **Correlation between the number of histidine residues modified by diethylpyrocarbonate and the residual enzyme activity.** The enzyme (2 μM) was incubated with 0.6 mM diethylpyrocarbonate in 0.02 M potassium phosphate buffer, pH 7.0, in the presence of 10% alcohol at 27 °C, and residual activity and the number of histidine residues modified were measured as described under "Materials and Methods." The data are presented in the form of Tsou plot for $i = 1$ (○), $i = 2$ (□), and $i = 3$ (△); details are given under "Results."

Number of Essential Histidine Residues—Histidine residues modified by diethylpyrocarbonate shows a characteristic absorption band with a maximum between 230 nm and 250 nm varying from protein to protein (14). The difference spectrum of epimerase after treatment with diethylpyrocarbonate showed a rapid development of a spectrum with an absorption maximum at 240 nm. Fig. 2 shows the relationship between

the loss of enzyme activity and the number of histidine residues modified. Extrapolation of the linear plot to zero enzyme activity shows that 6 residues are modified during complete inactivation. Since this method does not usually give the number of residues essential for activity (21, 22), the statistical method of Tsou (21) was used to calculate the number of essential histidine residues for inactivation.

If we assume that all the n-modifiable residues including i essential residues are approximately equally reactive toward the reagent and the modification of any of the essential residue results in complete inactivation, the relationship between residual activity against histidine modification will be as follows (21):

$$(A/A_0)^{1/i} = (n - m)/n \quad (1)$$

The number of the essential histidyl residue is that value of i which gives a straight line when residual activity (A/A_0) is plotted against m, the number of histidine residues modified. Epimerase activity was found to be dependent upon modification of 1 critical histidine (Fig. 2).

Specificity of Diethylpyrocarbonate Modification—Diethylpyrocarbonate has been shown to react in some proteins with residues other than histidine that include tryptophan, tyrosine, cysteine, etc. (14). As discussed later, both the intensity and the nature of the protein fluorescence spectrum of the epimerase remained unaffected (Fig. 4) on treatment with diethylpyrocarbonate. Tryptophan and tyrosine residues of epimerase, therefore, appear not to be modified by diethylpyrocarbonate. Hydroxylamine removes the carbethoxy group from diethylpyrocarbonate-modified histidyl residues but not from other amino acid residues like lysyl or cysteinyl residues (14). Epimerase inactivated by diethylpyrocarbonate could recover 70% of its native activity in the presence of hydroxylamine (Table I), thus excluding the possibility of involvement of lysyl or sulfhydryl groups of cysteine in diethylpyrocarbonate treatment. Increasing the time of reactivation normally did not enhance the activity any further. This significant reversal of activity upon treatment with hydroxylamine also suggests that a dicarbethoxy derivative of histidine is not formed upon modification of epimerase with diethylpyrocarbonate. In such a situation, treatment with hydroxylamine would have resulted in ring cleavage at histidine (14) with a concomitant irreversible loss in activity of the enzyme.

Finally, the effect of pH on the inactivation by diethylpyrocarbonate was examined in potassium phosphate buffer at the pH range of 6.4–8.0 where specifically histidyl residues react (14). The plot of apparent first order rate constants obtained at different pH against varying pH values gave a typical titration curve with a pK_a value around 7.0 (data not shown) which is consistent with the pK_a of the histidyl imidazolium group in proteins.

TABLE I

Reversal by hydroxylamine of diethylpyrocarbonate inhibition of UDPglucose 4-epimerase activity

Epimerase (0.6 mg/ml) was treated with 1.7 mM diethylpyrocarbonate in 0.02 M potassium phosphate buffer, pH 7.0, at 27 °C. At the end of the incubation period, 5 μl of modified enzyme was transferred to 25 μl of 0.05 M hydroxylamine, and the pH was adjusted to 7.5 and incubated at the same temperature. Control enzyme without diethylpyrocarbonate was kept under identical conditions, and the percentage of activity during reactivation was calculated by comparison with the activity in the control tube. Reactivation time was 1 h.

Treatments	Activity
	%
Control enzyme	100
Diethylpyrocarbonate-modified enzyme	<10
Hydroxylamine-treated enzyme	70

Some investigators have demonstrated that treatment of free adenosine with excess diethylpyrocarbonate results in a ring-opening reaction of the imidazole moiety of adenosine as well as reaction of the amino group of adenosine (23). In our case, treatment with diethylpyrocarbonate had no effect on the adenine moiety of bound NAD and inactivation of the enzyme was, therefore, not due to any such nonspecific modification of the coenzyme. This was demonstrated by the following experiment. One milligram (8 nmol) of the enzyme was taken in 0.3 ml of 0.02 M potassium phosphate buffer, pH 7.0, and incubated with 10 mM diethylpyrocarbonate for 90 min at 27 °C to ensure complete modification. The control enzyme sample containing ethanol only was kept under identical conditions. Both the control and the modified enzyme were heated at 70 °C for 15 min to dissociate NAD from the enzyme surface (24) and centrifuged to remove denatured protein. Concentrations of NAD in the supernatants were estimated with alcohol and yeast alcohol dehydrogenase. The assay mixture contained in a total volume of 1 ml, 100 μmol glycylglycine/sodium hydroxide buffer pH 8.8, 2% ethanol, 0.3 unit of alcohol dehydrogenase, and 0.3 ml of supernatant. The progress of the reaction was followed by the increase in absorbance at 340 nm. About 90% NAD was found to be present in unmodified form in diethylpyrocarbonate-modified enzyme.

Structural Integrity of the Inactivated Enzyme—The active holoenzyme from *K. fragilis* is a dimeric protein and contains 1 mol of NAD per mol of the dimer for catalytic activity (25, 26). Treatment with modifying reagents may result in a loss of activity if the dimer is dissociated or the coenzyme is ejected from the enzyme surface. Such situations were observed for the enzyme on treatment with *p*-chloromercuribenzoate (25, 26) or on controlled exposure to heat (24). In our case, the native enzyme after complete inactivation with diethylpyrocarbonate was found to retain its original holoenzyme structure. The diethylpyrocarbonate-modified enzyme showed the same elution profile as the native enzyme on a calibrated Sephacryl 200 column and also showed nearly identical mobility on native polyacrylamide gel electrophoresis (data not shown). The coenzyme was also not dissociated from the apoenzyme as the stoichiometric amount of NAD could be demonstrated to be associated with the diethylpyrocarbonate-inactivated enzyme after it was passed through a spin column by the method of Maniatis *et al.* (27) (data not shown).

Fluorescence Energy Transfer Phenomenon and Location of the Histidine Residue—The kinetic correlation between the loss of enzyme activity and modification of 1 histidine residue prompted us to investigate the physical location of the residue on the enzyme surface. Neither the substrates nor UMP, a powerful competitive inhibitor (28), could provide any protection against inactivation by diethylpyrocarbonate. The essential histidine residue was, therefore, probably not located in the substrate binding region of the active site. This study was, however, considerably facilitated by the discovery of an energy transfer phenomenon between the protein fluorophore(s) and the coenzyme fluorophore on the enzyme surface. One of the most interesting and important features of the *K. fragilis* enzyme is its unusual coenzyme fluorescence due to the bound NAD at the active site of the enzyme (2). Free NAD in an aqueous environment is, however, nonfluorescent. The coenzyme fluorescence spectrum broadly resembles the spectrum of free NADH although the exact chemical nature of the fluorophore remains unknown. This characteristic fluores-

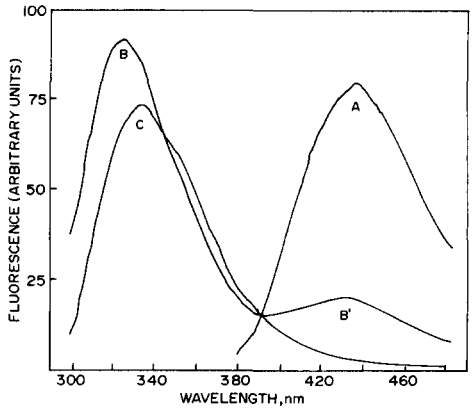

FIG. 3. **Fluorescence energy transfer in native epimerase.** For this experiment, 85 μg of epimerase (0.68 nmol) in 100 μl of 0.05 M phosphate buffer, pH 7.2, was taken in a cylindrical cuvette, and protein and coenzyme fluorescence spectra were recorded on excitation at 290 nm and 353 nm, respectively. Two hundred and fifty micrograms of lysozyme present in 100 μl of the above buffer were used for comparison. *A*, coenzyme fluorescence spectrum of epimerase; *B*, protein fluorescence spectrum of epimerase; *B'*, coenzyme fluorescence of epimerase on excitation at 290 nm due to the transfer of energy; *C*, protein fluorescence spectrum of lysozyme.

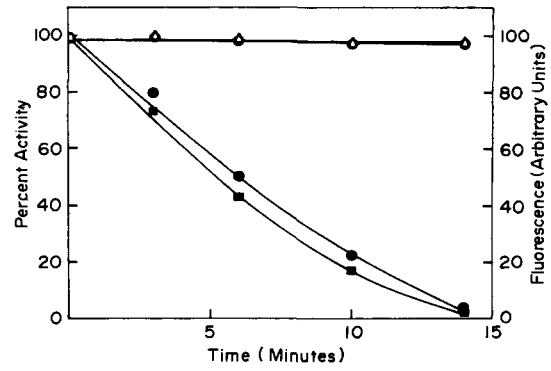

FIG. 4. **Relationship between inactivation of epimerase by diethylpyrocarbonate with energy transfer, protein fluorescence, and coenzyme fluorescence.** The coenzyme fluorescence (excitation, 353 nm; emission, 435 nm), protein fluorescence (excitation, 290 nm; emission, 335 nm), and energy transfer spectrum (excitation, 290 nm; emission, 435 nm) of the enzyme solution (0.15 mg/ml) in 0.02 M potassium phosphate buffer, pH 7.0, was recorded, and initial enzyme activity was assayed. The enzyme was next incubated with 3.8 mM diethylpyrocarbonate at 27 °C. Concentration of ethanol was 1%. At different time periods, the fluorescence spectra were followed, and the corresponding enzyme activities were measured by removing aliquots from the enzyme solution. The intensities of protein (○) and coenzyme fluorescence (△), the loss of efficiency of energy transfer (●), and loss of activity at different stages of diethylpyrocarbonate modification (■) are plotted against time of incubation with the reagent.

cence due to the pyridine moiety of the coenzyme can serve as a direct monitor of the changes at the active site and has been exploited by us to identify active amino acid residues in the catalytic regions of the active site (29, 30). We found that a small but significant portion of the protein fluorescence energy of the enzyme is transferred to the coenzyme fluorophore and is emitted at higher wavelengths. Fig. 3 shows the main features of the fluorescence properties of the native enzyme from *K. fragilis*. When the protein molecule was excited at 353 nm, a characteristic fluorescence with emission maximum at 435 nm due to the pyridine fluorophore was observed (*A*). When the enzyme was excited at 290 nm, the fluorescence emission spectrum exhibited two maxima with peaks at 330 nm (*B*) and 435 nm (*B'*), respectively. The peak at 330 nm is the typical fluorescence due to tryptophan and tyrosine residues, whereas the second peak resembles the coenzyme fluorescence. Since the absorption spectrum of NADH overlaps greatly with the protein fluorescence spectrum, the appearance of the second peak in the region of coenzyme fluorescence indicates the transfer of energy from 1 or more aromatic residues of the coenzyme fluorophore. Lysozyme, a typical protein, was used as control, and, as expected, did not show any other peak besides the usual protein fluorescence peak when excited under identical conditions (*C*). Employing *N*-bromosuccinimide, a modifying reagent for tryptophan under appropriate conditions, we have evidence to suggest that 2 tryptophan residues act as donors for this energy transfer process.[1] We observed that on modification of the native enzyme with diethylpyrocarbonate both the protein fluorescence and coenzyme fluorescence remained unaffected, but an exceedingly good kinetic correlation was obtained between the loss of activity and the loss of the energy transfer process (Fig. 4). Apparently, local conformational change in the vicinity of the catalytic region caused by modification of the essential histidine residue leads to an improper orientation between the donor and acceptor pyridine fluorophores that results in abolition of the energy transfer phenomenon.

A structural change in the catalytic region of the active site was further evident when an attempt was made to reduce bound NAD of the diethylpyrocarbonate-modified enzyme by sodium borohydride. Reduction by borohydride results in greatly enhanced fluorescence due to the formation of the catalytically abortive enzyme-NADH complex which is stabilized by 5'-UMP (2, 7). After diethylpyrocarbonate modification, bound NAD of the enzyme could not be reduced (Fig. 5) even when very high concentrations of the reagents were used. Obviously, treatment with diethylpyrocarbonate had rendered the coenzyme completely unreactive to sodium borohydride.

DISCUSSION

Diethylpyrocarbonate is a fairly specific reagent for histidine residues in the pH range 6.0–7.5 and has been used widely for the modification of histidine residues in various proteins (14, 15). Present investigation shows that UDPglucose 4-epimerase from *K. fragilis* was rapidly inactivated by diethylpyrocarbonate. Kinetic analysis by the method of Levy *et al.* (20) and by the method developed by Tsou (21) both indicate that modification of 1 essential histidine residue is responsible for the loss in catalytic activity of epimerase (Figs. 1 and 2). The pK_a value calculated from the pH dependence of inactivation and specific reversal of inhibition by hydroxylamine (Table I) suggest that the essential amino acid residue modified by diethylpyrocarbonate at pH 7.0 is a histidyl residue. Confirmation by amino acid analysis of the modification of the histidyl residue is not possible because *N*-carbethoxyimidazole is unstable under acid and alkaline conditions (14). The possibility of involvement of other aromatic amino acids in diethylpyrocarbonate modification was excluded by the unaltered protein fluorescence characteristics even after complete inactivation of the enzyme. Substantial reversal of activity by hydroxylamine eliminates the involvement of other susceptible amino acid residues like lysine or cysteine in diethylpyrocarbonate inactivation of the enzyme.

Although only 1 histidine residue was found to be essential for activity, 6 histidyl residues were modified by the time complete inactivation was achieved by diethylpyrocarbonate

[1] S. Mukherji and A. Bhaduri, unpublished observations.

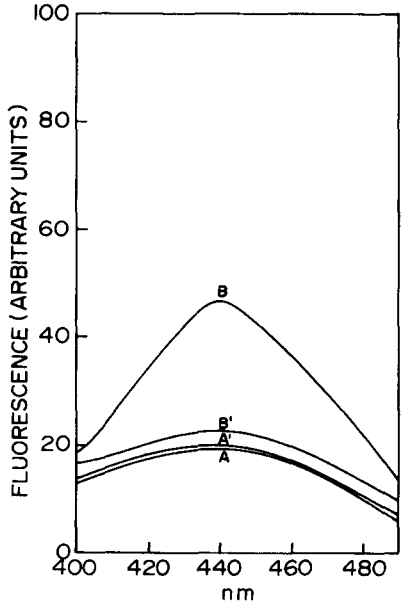

FIG. 5. **Reduction of NAD in diethylpyrocarbonate-modified enzyme.** The enzyme (0.8 mg/ml) was incubated with 3.7 mM diethylpyrocarbonate at 27 °C in 0.02 M potassium phosphate buffer, pH 7.0. To the control enzyme, only ethanol was added. After complete inactivation of the treated enzyme, excess reagent was removed by centrifuge column method of Maniatis *et al.* (27). To both the control and modified enzyme, 1 mM 5′-UMP and 20 mM sodium borohydride were added, and the fluorescence of the solutions on excitations at 353 nm was recorded. *A*, control enzyme; *B*, control enzyme + 5′-UMP (1 mM) + sodium borohydride (20 mM); *A′*, modified enzyme; *B′*, modified enzyme + 5′-UMP (1 mM) + sodium borohydride (20 mM).

treatment (Fig. 2). This, however, did not lead to any major change in the quarternary structure of the holoenzyme. This is evident from the following observations. The dimeric structure of the enzyme or the association of the coenzyme to the apoenzyme was retained and the fluorescence spectrum of both the protein and the coenzyme fluorophore remained unaffected even after complete loss of activity.

The histidine residue, essential for activity, is almost certainly not situated at the substrate binding region of the active site. Failure of the substrate or UMP, the competitive inhibitor, to afford any protection is evidence in this direction. This situation is in contrast to our earlier observation with arginine-modifying reagents (31). In these cases, both substrates and UMP provided complete protection, and the essential arginine residue was placed at the substrate binding region (31). The essential histidine residue seems to be located in close proximity to the coenzyme binding region of the active site. This is inferred from the fact that even though the chemical nature of the coenzyme fluorophore remains unaffected, the coenzyme is not amenable to chemical reduction after diethylpyrocarbonate modification (Fig. 5). The effective abolition of energy transfer phenomenon on complete inactivation, without any loss of protein and coenzyme fluorescence (Fig. 4), also indicates a local conformational change in the vicinity of a coenzyme binding region that possibly leads to improper orientation between the two fluorophores. Our modification experiments with diethylpyrocarbonate have thus uncovered the necessity for the presence of an unmodified histidine residue during normal catalysis. The specific mode of participation of this residue in the activity of this enzyme remains to be elucidated.

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