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# Chemical modification of the α-amylase of *Bacillus caldovelox* with diethyl pyrocarbonate: Evidence for an essential histidine at the active site

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## SUMMARY

The α-amylase of *Bacillus caldovelox* is inactivated by diethyl pyrocarbonate at pH 6.6 and 20 °C by a monomolecular reaction with a second-order rate constant of 41.7 $M^{-1} \cdot min^{-1}$. The rate of inactivation increases with decreasing pH, suggesting participation of an amino acid residue with a p*K*a of 6.6. The increase in absorbance at 240 nm, unchanged absorbance at 280 nm and reactivation in the presence of hydroxylamine suggest the participation of a histidine residue. Statistical analyses of inactivation suggest that only one histidine residue is essential for activity. Substrate afforded complete protection against inactivation, indicating the involvement of the histidine residue at the active site of the enzyme.

## INTRODUCTION

α-Amylases (α-1,4-D-glucan glucanohydrolase, EC 3.2.1.1 endoamylase) catalyse the conversion of starch to malto-oligosaccharides by an endoacting hydrolytic mechanism [7].

Industrial starch conversion involves high temperature liquefaction by thermostable bacterial amylases followed by low temperature saccharification by fungal amylases. Considerable commercial interest has recently developed in the production of high levels of specific malto-oligosaccharides by certain saccharifying bacterial amylases [8]. The caldoactive bacterium *Bacillus caldovelox* produces a thermostable saccharifying α-amylase [2]. This novel amylase produces higher levels of maltohexaose from starch than any amylase reported to date [2,8].

Despite the obvious industrial importance of bacterial liquefying and saccharifying amylases little is known about their active site and mechanism of action. In contrast, recent X-ray and sequence work, together with genetic engineering and chemical modification analyses of the saccharifying amylases of *Aspergillus niger*, *Aspergillus oryzae* [4] and porcine pancreatic α-amylase [5] have yielded considerable information about the active sites and similarity of these enzymes.

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Chemical modification of enzymes is a powerful and probing technique and has been successfully used to investigate the active sites of the α-amylase of *A. oryzae* [9] and the amyloglucosidase of *A. niger* [19]. Analyses of porcine pancreatic [5] and *A. oryzae* [4,13] α-amylase have shown the involvement of histidine in their active sites. The primary calcium binding site in *Aspergillus* amylases is coordinated by eight ligands, one of these is the carbonyl oxygen of Glu210 in *A. niger* at a distance of 2.42 Å. In *A. oryzae* Glu210 is replaced by His210 and the corresponding distance is 2.43 Å [4]. There is a lack of similar data on the bacterial amylases preventing complete comparisons with their fungal equivalents and it is therefore unknown whether the active site is conserved in all amylases. However, it has been proposed [16,18] that not only do all α-amylases examined, both fungal and bacterial, display the same basic super-secondary structure with $(\alpha/\beta)_8$ barrel but that carbohydrases with activity on α-1,4- and α-1,6-glucosidic linkages, share key structural features in their active sites.

This paper presents chemical modification analysis which suggests the involvement of a histidine residue at the active site of the thermostable high maltohexaose-producing α-amylase of *Bacillus caldovelox* [2]. A consensus would therefore appear to be emerging, which suggests the involvement of histidine in catalysis of both the fungal and bacterial, as well as the mammalian amylases examined to date.

## MATERIALS AND METHODS

Starch and 3,5-dinitrosalicylic acid (DNS) were purchased from British Drug House. Dextrin 01913 was purchased from CPC (Irl) Ltd. Bicinchoninic acid, diethylpyrocarbonate (DEP), hydroxylamine hydrochloride and 4-morpholine-ethanesulphonic acid (MES) were purchased from Sigma (St. Louis, MO, U.S.A.). Hydroxylamine was freshly prepared by equimolar neutralisation of the free acid. All other chemicals were of analytical grade. Enzfitter, a non-linear regression data analysis program was used for all linear and non-linear regression analyses [10].

*Microorganism*

*Bacillus caldovelox* DSM 411 was obtained from Deutsche Sammlung von Mikroorganismen, and maintained as previously described [2].

*Enzyme assay*

α-Amylase was assayed by adding 0.5 ml of enzyme to 1% (w/v) soluble starch in 0.1 M acetate buffer, pH 4.5 and incubating at 40 °C for 30 min. The reaction was stopped and the reducing sugars determined using dinitrosalicylic acid (DNS) according to the method of Bernfeld [3]. An enzyme unit is defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate, per 30 min at 40 °C.

*Protein assay*

Protein was determined by a modified version of the bicinchoninic acid method of Smith et al. [17].

*Enzyme production and purification*

Enzyme was produced and purified to homogeneity as previously described by this laboratory [2].

*Preparation and standardization of DEP solutions*

DEP was diluted with cold ethanol immediately prior to use. The concentration of DEP in this solution was assayed by adding 10 μl to 3 ml of 10 mM imidazole (pH 7.0) and monitoring the Ultra Violet absorbance at 230 nm in an LKB Ultrospec UV II spectrophotometer. The concentration of DEP was calculated from the extinction coefficient (3000 $M^{-1} \cdot cm^{-1}$) of the N-carbethoxyimidazole [15] produced according to Beer's law.

*Reaction of DEP with α-amylase*

A known quantity of pure enzyme in 0.1 M MES buffer (pH 6.6) was incubated with DEP (0.4 mM final concentration) at 20 °C. The final concentration of ethanol in the reaction never exceeded 2.5% (v/v). Aliquots were removed at appropriate time intervals and diluted 100-fold in 0.1 M acetate buffer (pH 4.5). This solution was then assayed for activity.

*Statistical analysis of modification reactions*

The pseudo-first-order modification rate constants ($k_{obs}$) were calculated from the slope of the plots of logarithm of residual activity against reaction time. The second-order rate constant was obtained from the slope of the plots of pseudo-first-order rate constants against DEP concentration. The order or molecularity of the reaction was calculated from the slope of plots of minus the logarithm of pseudo-first-order rate constants against minus the logarithm of DEP concentration [11].

*pH dependence of pseudo-first-order constants ($k_{obs}$)*

Enzyme was modified with DEP (final concentration 0.4 mM) over a range of pH values (5.6–7.9) in 0.1 M MES buffer. The $pK_a$ of the modified residues was obtained from a plot of the pseudo-first-order rate constants, at each pH, against pH by non-linear regression analysis.

*Carbethoxylation of histidine residues*

A known quantity of pure enzyme was modified with DEP as previously described. The progress of the reaction was monitored spectrophotometrically by measuring the time-dependent increase in absorbance at 240 nm against a blank containing only buffer and enzyme. The concentration of carbethoxyhistidine formed was calculated from the extinction coefficient (3200 $M^{-1} \cdot cm^{-1}$) [15] according to Beer's law. Determination of the number of residues modified per enzyme molecule was performed according to the method of Tsou [20].

*Determination of essential catalytic residues*

A plot of residual activity against the residues modified with DEP yielded a sigmoidal curve which when extrapolated yielded the number of modified residues ($n$). Assuming all residues are equally reactive, then the number of modified residues ($m$), at any stage of the reaction, is given by the equation:

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

The number of essential residues ($i$), was the value which produced a linear graph from the experimental data according to equation (1) [20].

*Recovery of enzyme activity*

Enzyme was modified as previously described, with 0.4 and 1.0 mM DEP (final concentration) for 0, 30 and 60 min and residual activity measured. This modified enzyme was subsequently incubated with 1.0 M hydroxylamine (pH 7.0) for 30 min at 20 °C and the residual

activity measured. Controls consisted of unmodified enzyme and were treated identically.

## RESULTS

*Reaction of α-amylase with DEP*

α-Amylase modification with DEP resulted in enzymic inactivation which was both time- and modifier-concentration-dependent. Prolonged incubation resulted in a complete loss of activity. Results obtained from the linearity of semi logarithm plots of residual activity against time, suggest that the inactivation follows first-order kinetics, within the concentration range of DEP used (Fig. 1).

Pseudo-first-order rate constants ($k_{obs}$) were obtained from the slope of the plots of log of the residual activity against reaction time by linear regression analyses (Fig. 1). The relationship between the observed pseudo-first-order rate constant ($k_{obs}$) and the concentration of DEP can be described by the equation:

$$k_{obs} = k[DEP]^n \quad (2)$$

where ($k$) is the second-order rate constant with respect to DEP concentration and ($n$) is the reaction order.

The molecularity of the reaction ($n$) was obtained from the slope of minus the log of $k_{obs}$ against minus the log of

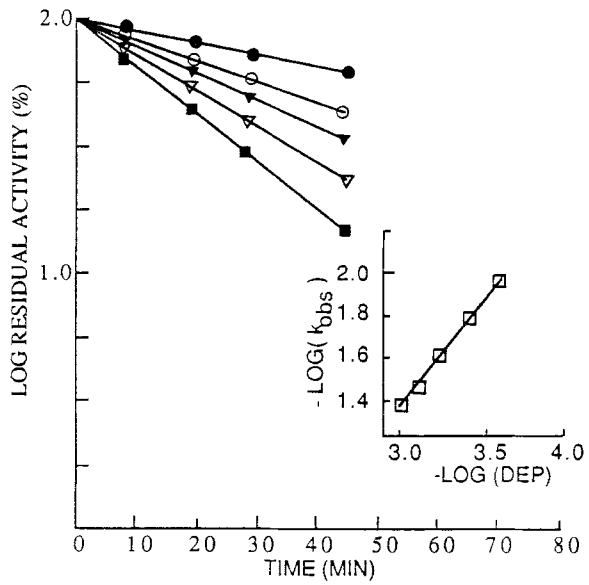


Fig. 1. Effect of DEP on the α-amylase of *Bacillus caldovelox*. Enzyme was incubated with DEP: 0.2 mM (●), 0.4 mM (○), 0.6 mM (▼), 0.8 mM (▽) and 1.0 mM (■). Inset: apparent order of reaction with respect to modifier concentration. Pseudo-first-order constants calculated from Fig. 1.

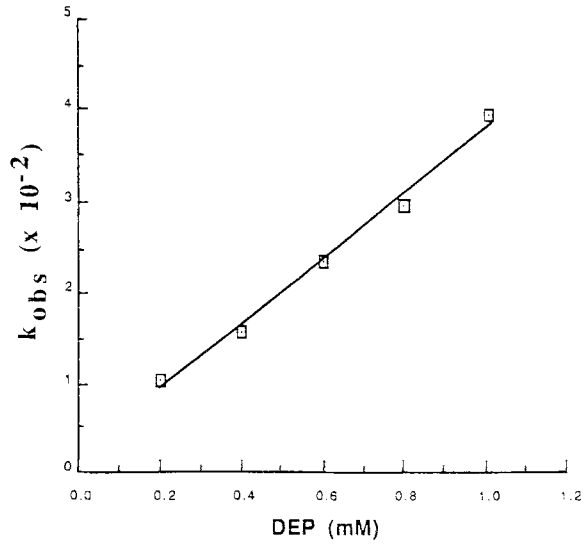


Fig. 2. Effect of DEP concentration on the observed pseudo-first-order rate constants of inactivation of the α-amylase of *Bacillus caldovelox*.

DEP concentration (Fig. 1, inset). The value of 0.81 obtained suggests a monomolecular reaction mechanism.

A second-order rate constant of 41.7 $M^{-1} \cdot min^{-1}$ was obtained from the slope of the linear plots of pseudo-first-order rate constants against DEP concentrations (Fig. 2).

*Modification of histidine residues*

Modification of the α-amylase of *B. caldovelox* with DEP was accompanied by an increase in absorbance at 240 nm, with no change in the absorbance at 280 nm (not shown). These results are consistent with the characteristic increased absorbance at 240 nm of carbethoxyhistidine, which is formed by the DEP modification of histidine. Tryptophan and tyrosine, the amino acids responsible for the characteristic 280 nm absorbance of proteins, can also be modified with DEP but with a concomitant decrease in absorbance at 280 nm. The unchanged absorbance in the 280 nm region of the enzyme absorbance spectrum therefore negates the possibility of tryptophan and tyrosine modification [14,15].

*Reversibility of enzyme inactivation*

DEP can potentially modify lysine and sulfhydryl as well as histidine residues. However, unlike histidine modification, the reactions with both lysine and sulfhydryl residues are irreversible. Therefore to differentiate between histidine and non-histidine modification the reversibility of the reaction was examined (Table 1). Incubation of partially inactivated enzyme with hydroxylamine restored enzymic activity. The restoration of

TABLE 1

Effect of incubation, of the partially inactivated, DEP-modified α-amylase, with hydroxylamine. Enzyme modified with DEP for 0, 30 and 60 min was incubated with hydroxylamine and residual activity measured.

| Time (min) | DEP (mM) | Residual activity (%) | % Activity recovered with 1.0 M $NH_2OH$ (30 min) |
|---|---|---|---|
| 0 | 0 | 100 | 100 |
| 30 | | 100 | 104 |
| 60 | | 96 | 97 |
| 0 | 0.4 | 100 | 100 |
| 30 | | 84 | 105 |
| 60 | | 64 | 97 |
| 0 | 1.0 | 100 | 100 |
| 30 | | 35 | 85 |
| 60 | | 25 | 75 |

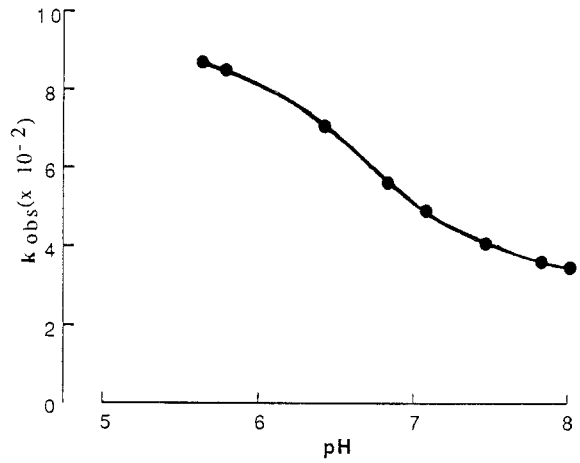


Fig. 3. pH-dependence of the observed pseudo-first-order rate constants of inactivation of the α-amylase of *Bacillus caldovelox*, modified with DEP.

activity was only complete for enzyme modified by low concentrations of DEP. This suggests that no excess of modifier existed at this concentration and that the inactivation was not due to denaturation processes. Restoration of activity also indicates that modification of either histidine or tyrosine, but not lysine or thiol residues occurred [15] and tyrosine participation had already been eliminated. Enzyme partially inactivated with 1.0 mM DEP could not be fully reactivated despite prolonged incubation with hydroxylamine (data not shown). This would suggest that modification of both histidine (reversible) and non-histidine (irreversible) residues occurred, and that the non-histidine residues were responsible for the unrecoverable moiety of activity lost. Alternatively it is also possible that some form of limited irreversible denaturation process is occurring in conjunction with histidine modification. As the modification was found to be histidine-specific at 0.4 mM DEP this concentration was used in all subsequent experiments.

*Effect of pH on enzyme modification*

The pH dependence of amylase modification with DEP was evaluated from a plot of the pseudo-first-order rate constants calculated over a pH range of 5.6 to 7.9 against the pH of modification (Fig. 3). However, it must be noted that at pH values above 7.0 available DEP may be reduced [12]. A $pK_a$ of 6.6 was calculated from the data by non-linear regression analysis. This $pK_a$ is consistent with the normal range of values for histidine in proteins.

*Catalytically essential histidines*

A plot of residual activity against histidine residues modified, yielded a sigmoidal curve (data not shown). Initially two histidines were modified with minor inactivation. Subsequent modification greatly inactivated the enzyme. Extrapolation indicated that modification of 8 histidine residues was necessary for complete inactivation. Extrapolating only the fast phase of the curve indicated that of these 8 modifiable residues only three are critical for catalytic activity. This method has been criticised for over-estimating the number of residues involved [15]; it does, however, provide an estimate of the total number modifiable. Taking account of this estimate of the total number of modifiable residues, a more accurate and

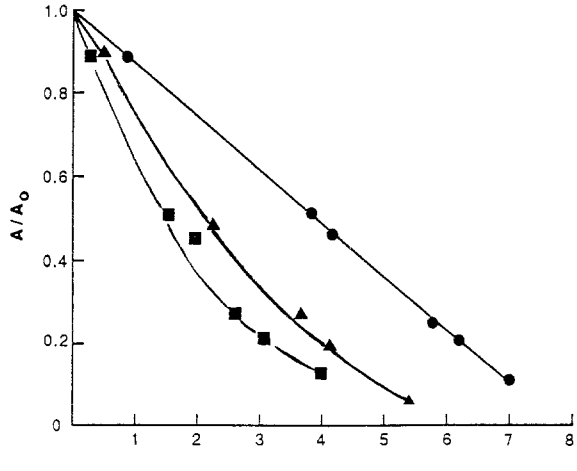


Fig. 4. Relationship between residual activity and the number of histidine residues modified. Experimental data were transformed according to equation (1), with $n = 8$ and $i = 1$ (●), 2 (▲) and 3 (■).

reliable statistical method was developed by Tsou [20]. This analysis attempts to fit the experimental data to a linear equation (1). According to this analysis, a linear-fit of the experimental data to equation (1), was found when $i = 1$ (Fig. 4), suggesting that only one histidine was essential for activity.

*Protection of α-amylase against inactivation*

The α-amylase of *B. caldovelox* was completely protected against modification with DEP by pre-incubation with Dextrin 01913 (a low viscosity starch hydrolysate), despite the level of DEP (0.2–1.0 mM) used (data not shown). This result suggests that the critical histidine residue, essential for activity, is located at a locus on the surface or in a cleft of the protein which is blocked by substrate during catalysis, thus affirming the theory that the role of the essential residue may be in catalysis itself rather than being involved in maintenance of the general structural integrity of the enzyme.

## DISCUSSION

DEP displays selectivity in reacting with histidine in proteins within the pH range 6.0 to 7.0. However, when in excess, reactions with tryptophan, tyrosine, lysine and cysteine residues have been reported [15].

The α-amylase of *B. caldovelox* is reversibly inactivated by modification with DEP, within the pH range 6.0 to 7.0. The hypothesis that histidine is the only residue modified is supported by a number of confirmatory results. Firstly the increased absorbance at 240 nm is consistent with histidine modification, while the unchanged absorbance at 280 nm negates the involvement of either tryptophan or tyrosine [15]. Furthermore, the complete restoration of enzymic activity by incubation with hydroxylamine conclusively facilitates the argument that only histidines were modified, as this reversal reaction is not possible with lysine or sulfhydryl residues [14]. This complete restoration also proves that no gross distortion or denaturation occurred, justifying the concentration of DEP used. The experimentally obtained p*K*a of 6.6 is consistent with values for histidine in RNA polymerase [6] and uridine phosphorylase [1] from *Escherichia coli.* However, the accuracy of this determination may be influenced by the instability of the DEP reagent at pH values above 7.0 [12].

The inactivation of the enzyme during modification proceeded with first-order kinetics, however, the modification of histidine residues proceeded at two rates. Initially two residues were modified with only a minor affect on activity, while the subsequent modification dramatically inactivated the enzyme. Further analyses suggested that only one histidine was essential for activity. Modification of the α-amylase of *A. oryzae* yielded similar results, with only 1 of the 6 modified histidine residues playing an important role in catalysis [9].

The complete protection afforded to the enzyme, in the presence of substrate, further suggests that the essential histidine modified is at a site involved in the catalytic function of the enzyme. This is in agreement with the involvement of histidine in *A. oryzae* [4] and porcine pancreatic α-amylase [5] and further supports the view that regardless of source, α-amylases share key features at the active site.

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