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MODIFICATION OF ENOYL-CoA HYDRATASE USING DIETHYL PYROCARBONATE

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Diethyl pyrocarbonate inactivates enoyl-CoA hydratase (L-3-hydroxyacyl-CoA hydro-lyase, EC 4 2 1 17) with a second-order rate constant of 1 3 M^{-1} s^{-1} Partial protection is given against inactivation by the substrate analogue acetoacetyl-CoA The single histidine per enzyme subunit is completely modified at a rate considerably faster than inactivation, and enzymatic activity is not restored by treatment with hydroxylamine No tyrosine, cysteine or tryptophan residues are modified by diethyl pyrocarbonate However, out of the 22 amino groups per subunit, 2–5 groups do react with diethyl pyrocarbonate, as shown by difference titration with methyl[1-^{14}C]-acetimidate Destruction of the N-terminal serine residue by periodate oxidation lowers, but does not abolish enzymic activity Experiments using ^{3}H-labelled diethyl pyrocarbonate show that the loss of 85% of the original activity is accompanied by the incorporation of approx three carbethoxy groups One amino acid residue reacts much faster than the others, and is not essential for activity Of the next two groups reacting, one is apparently essential for activity Modification with diethyl pyrocarbonate does not lead to any gross changes in the structure of the enzyme These experiments taken together show that, in contrast to other hydratases, histidine is not involved in the catalytic mechanism of enoyl-CoA hydratase, and suggest that a single lysine residue is important for activity

Introduction

Enoyl-CoA hydratase (L-3-hydroxyacyl-CoA hydro-lyase, EC 4 2 1 17), an enzyme in the fatty acid oxidation cycle, catalyses the reversible hydration of 2,3-unsaturated acyl-CoA compounds to the corresponding (3*S*)-hydroxy derivatives [1–3] The enzyme is extremely efficient, with a k_{cat}/K_m for the hydration of crotonoyl-CoA of 2 8 10^8 M^{-1} s^{-1} [4], close to the diffusion-controlled limit Unlike most hydratases, enoyl-CoA hydratase catalyses the *syn* addition of water to a carbon-carbon double bond [5,6] Relatively little is known about specific amino acid residues which are involved in the catalytic mechanism It has been proposed that a sulfhydryl group is essential for catalysis, since the hydratase can be inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) and other thiol-specific reagents [3] However, further work [7,8] has shown that inhibition by 5,5′-dithiobis(2-nitrobenzoic acid) can probably be attributed to a conformational change caused by the modification of internal thiol groups, and that cysteine is not directly involved in the hydration of substrate Histidine residues have been implicated in the catalytic mechanism of at least three other hydratases [9–11]

We report here the modification of enoyl-CoA hydratase by diethyl pyrocarbonate, which, although useful for the selective modification of histidine, can also react with lysine, tyrosine, tryptophan and cysteine [12] Diethyl pyrocarbonate rapidly inactivates the enzyme, modifying a small number of resi-

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dues, and causing no significant alteration either in the fluorescence properties of the hydratase or in its sedimentation coefficient

Materials and Methods

Enzymes Enoyl-CoA hydratase was prepared from bovine liver using the method of Stern [3] as modified by Steinman and Hill [8], except that the acetone precipitation step was omitted and the enzyme was eluted from a CM-cellulose column at pH 6 0 before crystallization Freshly-recrystallized enzyme was used for the modification experiments It had a specific activity of 1 800 kat/mol, when assayed with crotonoyl-CoA (10 μM) as substrate [1], migrated as a single band during SDS-polyacrylamide gel electrophoresis [13], and was homogeneous during analytical ultracentrifugation

Hydratase concentrations were determined from absorbance at 280 nm, using $A_{280}^{0.1\%} = 0\ 58$ [14] and a molecular weight of 164000 for the hexameric enzyme [14] Pepsin (ex hog mucosa), three times recrystallized, was from Sigma Chemical Co (St Louis, MO, U S A)

Chemicals Crotonoyl-CoA, acetoacetyl-CoA and acetyl-CoA (as their lithium salts) were obtained from P-L Biochemicals Inc , through International Enzymes Ltd (Windsor, Berks , U K), and Coenzyme A (free acid) from Boehringer Mannheim (Lewes, Sussex, U K) Sephadex G-25 was obtained from Pharmacia Fine Chemicals Ltd (Uppsala, Sweden) Triton X-100 and 5-(*N,N*-dimethylamino)-1-naphthalenesulphonyl(dansyl)chloride were from Sigma Chemical Co (St Louis, MO, U S A), and diethyl pyrocarbonate was from Aldrich Chemical Co (Gillingham, Dorset, U K) Polyamine TLC sheets were supplied by BDH Ltd (Poole, Dorset, U K) Guanidine hydrochloride was also from BDH, and was recrystallized twice from ethanol Generally-labelled [G-^{3}H]-diethyl pyrocarbonate was synthesised by the Radiochemical Centre (Amersham, U K), using a modification of the Wilzbach technique [15] The re-distilled product contained 100 μCi/mg, and was used in modification experiments without further preparation Methyl[1-^{14}C]acetimidate (0 116 Ci/mol) was a kind gift from Mr J Armstrong

Methods The specific radioactivity of the [G-^{3}H]-diethyl pyrocarbonate was measured by reacting excess reagent with pepsin at pH 4 in 100 mM sodium acetate buffer, for 1 h at 20°C Under these conditions, one amino acid residue of pepsin reacts cleanly with diethyl pyrocarbonate [16] The reaction mixture was passed through a column of (0 8 × 20 cm) Sephadex G-25 (fine) and protein-containing fractions were pooled Pepsin concentrations were determined from absorbance at 280 nm, using $A_{280}^{0.1\%} = 0\ 51$ [17] Radioactivity associated with the protein was measured by mixing samples with 10 vol toluene/Triton X-100 ((2 1) v/v) containing 2,5-diphenyloxazole (0 4%, w/v) and 1,4-bis(2-(5-phenyloxazolyl))-benzene (0 013%, w/v) and counting in an LKB model 1215 Rackbeta liquid scintillation counter Quench corrections were carried out using the sample channels ratio method, the counting efficiency for tritium being about 40% Two independent determinations gave values for the specific radioactivity of the diethyl pyrocarbonate of 4 2 and 4 6 Ci/mol, respectively, and the average value was used in calculations

Chemical modification with diethyl pyrocarbonate was performed as described in the figure legends Stock solutions of diethyl pyrocarbonate were prepared by dilution into ice-cold absolute ethanol The concentration of diethyl pyrocarbonate in these stock solutions was determined by reacting a portion with excess 10 mM imidazole-HCl buffer (pH 7 4) The concentration of *N*-carbethoxyimidazole formed was calculated from the increase in absorbance at 230 nm, assuming an extinction coefficient of 3 10^3 M^{-1} cm^{-1} [12]. The reagent has a half-life of 6 h at 0°C under these conditions [18] Samples for activity assays were diluted a 100-fold into cold 100 mM potassium phosphate, 1 mM EDTA, pH 7 2, containing 0 1 mg/ml bovine serum albumin Samples for tritium counting were precipitated with 10% (w/v) trichloroacetic acid and collected on glass fibre filters After extensive washing with trichloroacetic acid and then acetone, the filters were dried, placed in 5 ml toluene/Triton scintillation fluid and counted for radioactivity Since no more than 0 1 mg protein was ever loaded onto a filter, errors due to self-absorption should be small [19]

Hydroxylamine treatment of protein modified with diethyl pyrocarbonate was performed by adding 0 16 vol neutral, 2 M hydroxylamine directly to the reaction mixture [12] After 1 h at room tempera-

ture, solutions were desalted using a column (1 0 × 13 5 cm) of Sephadex G-25 (medium) and analysed for protein content and hydratase activity

Titration of sulfhydryl groups Titration of reactive thiol groups was carried out with 5,5′-dithiobis-(4-nitrobenzoic acid) [20] Enzyme solutions (0 25 ml, 25 μM in hydratase subunits) were mixed with 1 0 ml 125 mM Tris-HCl buffer (pH 8 0) containing 7 5 M guanidine hydrochloride, and reaction was initiated with 50 μl 22 mM 5,5′-dithiobis(4-nitrobenzoic acid), in 0 5 M Tris-HCl buffer (pH 8 0) The extent of the reaction was determined by measuring the increase in absorbance at 412 nm after 5 min

Titration of amino groups Titration of amino groups was carried out under denaturing conditions using methyl[1-^{14}C]acetimidate [21] Portions of reaction mixtures containing 2–5 mM diethyl pyrocarbonate and 100 μM hydratase subunits were quenched by mixing with 6 vol 8 3 mM imidazole buffer, and dialysed against 0 4% (w/v) ammonium bicarbonate After removal of ammonium bicarbonate by repeated lyophilization, each sample was dissolved in 100 mM *N*-ethyl morpholine/acetic acid buffer, pH 8 8, containing 6 M guanidine hydrochloride Protein concentration was estimated from A_{280} measurements, and methyl[1-^{14}C]acetimidate (1 1 mg/ml in the same buffer, containing 1 equiv NaOH) was added to a final concentration of 100 mM The solutions were left at 40°C for 4 h Duplicate samples from each solution were precipitated with 10% (w/v) trichloroacetic acid and prepared for scintillation counting as described above Quench correction was carried out by the external standards ratio method, and counting efficiency for ^{14}C was approx 90%

Periodate oxidation of N-terminal serine residue Oxidation of enoyl-CoA hydratase with sodium periodate was carried out essentially as described by Dixon [22] After 20 min at room temperature, excess periodate was destroyed by addition of KBH_4 and 2-mercaptoethanol was then added, to a final concentration of 75 mM The protein was desalted on a column (1 5 × 15 cm) of Sephadex G-25, equilibrated with 0 4% (w/v) ammonium bicarbonate, and samples taken for protein determination and assay of enoyl-CoA hydratase activity The presence of N-terminal serine was monitored, before and after modification by the dansylation procedure [23] Dansyl derivatives were separated by chromatography on polyamide thin layers [24]

Ultracentrifugal analysis Sedimentation velocity experiments were carried out using a Spinco Model E analytical ultracentrifuge equipped with schlieren optics, in a twin-sector cell with a Kel-F centrepiece The rotor speed was 52 640 rev /min, and photographs were taken every 8 min Apparent sedimentation coefficients were calculated using the method of Schachman [25] and corrected to $s_{20,w}$ values

Spectroscopic methods Fluorescence spectra were recorded at 10°C using an Aminco-Bowman spectrofluorimeter, equipped with a Xenon lamp Spectrophotometric measurements were carried out using either a Cary model 219 spectrophotometer or a Hilger-Watts spectrophotometer modified by the attachment of a Gilford monochromator

Results

Inhibition of enoyl-CoA hydratase by diethyl pyrocarbonate The effects of three concentrations of diethyl pyrocarbonate on hydratase activity are shown in Fig 1 The apparent initial rate of inactivation is directly proportional both to the concentration of enzyme (data not shown) and the concentration of diethyl pyrocarbonate A second-order rate constant $k = 1\ 3\ M^{-1}\ s^{-1}$ can be calculated from the data in Fig 1 The rate of loss of activity diminished after 10–15 min, and a second portion of diethyl pyrocarbonate was required to complete the inactivation This presumably reflects the fact that diethyl pyrocarbonate hydrolyses with a half-life of 9 min under these conditions [18]

Protection by substrate analogues against inactivation Inactivation of enoyl-CoA hydratase by diethyl pyrocarbonate in the presence or absence of a substrate analogue is shown in Fig. 2 Acetoacetyl-CoA, which has a K_{diss} of 20 μM, comparable to the K_m of crotonoyl-CoA [26], causes an initial reduction in the rate of hydratase activity loss, giving about 50% protection at a concentration of 200 μM However, acetyl-CoA, a poor competitive inhibitor of this enzyme [26] gives no protection at this concentration (data not shown) Side reactions between diethyl pyrocarbonate and the adenosine portion of CoA are possible [27] but cannot account for the observed difference between acetoacetyl-CoA and acetyl-CoA in these experiments

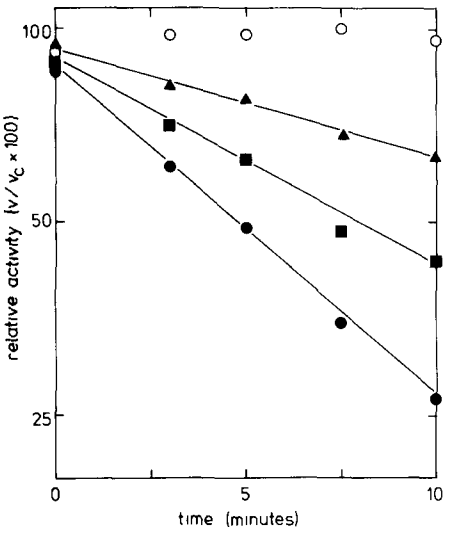

Fig 1 Semilog plot of changes in the activity of 25 μM enoyl-CoA hydratase in 50 mM potassium phosphate (pH 7 2)/0 5 mM EDTA, at 25°C, on reaction with either no (○), 0 5 mM (▲), 1 0 mM (■) or 1 5 mM (●) diethyl pyrocarbonate

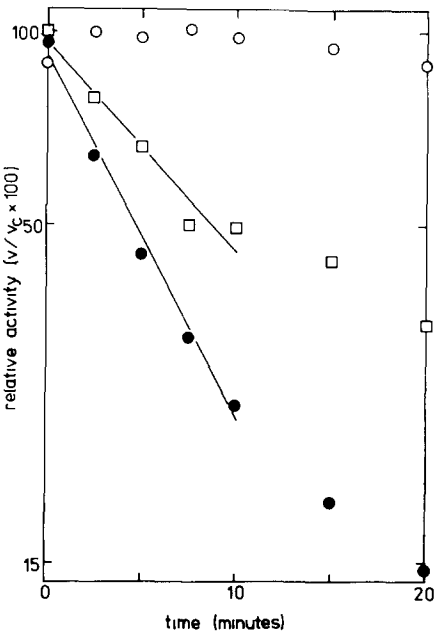

Fig 2 Inactivation of 25 μM enoyl-CoA hydratase in 50 mM potassium phosphate (pH 7 2)/0 5 mM EDTA, at 25°C Modification was carried out in the absence (●) of competitive inhibitor, or in the presence (□) of 200 μM acetoacetyl-CoA The control (○) retains essentially full activity over this period of time

Modification of histidine, tyrosine and cysteine residues To test for histidine or tyrosine modification by diethyl pyrocarbonate, difference spectra were taken at 240 and 278 nm An increase in absorbance at 240 nm, equivalent to the formation of one carbethoxy-histidine residue per subunit, was complete within 4 min, although 34% of the initial enzyme activity remained Addition of another portion of diethyl pyrocarbonate removed all residual activity but did not alter the absorbance at 240 nm The increase in absorbance at 240 nm was completely reversed by treatment with hydroxylamine, but this treatment did not restore enzyme activity A control solution treated with hydroxylamine retained 72% of its activity Since the hydratase contains one histidine residue per subunit [14], these experiments show that histidine is not involved in the catalytic mechanism No change in absorbance at 278 nm was observed during the reaction, indicating that tyrosine modification did not take place The number of free thiol groups was unchanged, as judged by titration with 5,5′-dithiobis(4-nitrobenzoic acid), after 10 min incubation with 1 5 mM diethyl pyrocarbonate, although the enzyme had lost 77% of its original activity

Modification of amino groups Enoyl-CoA hydratase, which had been inactivated by diethyl pyrocarbonate so that approx 15% of the original activity remained, consistently contained fewer free amino groups than untreated enzyme Titration with methyl[1-^{14}C]acetimidate [21] revealed that an average of 3 5 ± 1 5 amino groups per subunit (out of 22) had been modified by diethyl pyrocarbonate under these conditions The α-amino group of the N-terminal serine residue is apparently among the amino groups modified, since examination of inactivated enoyl-CoA hydratase by the dansylation procedure failed to reveal a free N-terminus However, when the N-terminal serine is completely destroyed by oxidation with periodate, the enzyme retains 20% of its original activity, so inactivation by diethyl pyrocarbonate is probably due to modification of the ε-amino groups of lysine residues

Inactivation of enoyl-CoA hydratase with tritiated

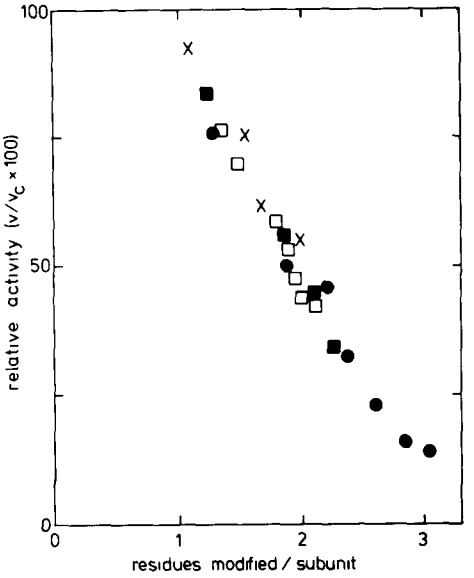

Fig 3 Correlation of inactivation of enoyl-CoA hydratase with the number of acid-stable carbethoxy groups introduced by diethyl pyrocarbonate 50 μM enoyl-CoA hydratase were incubated with 2 0 mM (•) or 2 1 mM (▪) [^{3}H]-diethyl pyrocarbonate in the absence of competitive inhibitor, or with 1 4 mM (□) or 2 1 mM (X) [^{3}H]-diethyl pyrocarbonate in the presence of 250 μM acetoacetyl CoA

diethyl pyrocarbonate The correlation between the degree of enzyme inactivation by tritiated diethyl pyrocarbonate and the incorporation of tritium label into acid-insoluble material is shown in Fig 3. After an initial lag during which 0 8 carbethoxy groups are incorporated per subunit without loss of activity, there is a steady inactivation which reaches 85% when approx three residues per subunit have reacted with diethyl pyrocarbonate Of the amino acid residues likely to be modified, histidine is least stable in acid [28] In control experiments, *N*-carbethoxyhistidine was found to have a half-life, at room temperature in 10% trichloroacetic acid, of about 2 h (see also Ref 29) Even though tritium incorporation was measured immediately after completion of the reaction, it is possible that the extent of modification of the histidine residue will have been underestimated by 20–30% Fig 3 also shows that the relationship between loss of activity and carbethoxylation of the enzyme is not affected by the presence of acetoacetyl-CoA, although the competitive inhibitor reduces the initial rate of inactivation

The data in Fig 3 were analysed by the algebraic method of Tsou [30,31] in which enzymic activity remaining (a) is related to the number of residues of the same type which have reacted (m), among them a number of essential residues (i) In this case, fast reaction of inessential residues is followed by reaction of a slower set, containing the essential group(s) The best straight-line fit was sought to the equation

$$a^{1/i} = \alpha + \beta m \qquad (1)$$

(where α and β are constants), by choosing alternative, integral values of i and m A satisfactory fit to the data was obtained by choosing $m = 2$ and $i = 1$, but a reasonably straight line was also obtained with $m = 3$ and $i = 2$ The improvement in fit by using a quadratic equation to describe the data [31] was 1 2-fold for $m = 2, i = 1$ and 1 1-fold for $m = 3, i = 2$.

Effects on enzyme structure of modification by diethyl pyrocarbonate The gross conformational properties of modified and unmodified enzyme were examined using sedimentation velocity experiments and fluorescence measurements Native and modified enoyl-CoA hydratase both sediment with coefficients of 8 7 S, in agreement with published values [14], indicating that the quaternary structure has not been affected by the modification Fluorescence measurements provide a sensitive measurement of changes in protein tertiary structure Enoyl-CoA hydratase fluoresces most strongly when excited at 278 nm and the maximum emission in the spectrum is at 330 nm After inactivation, the excitation frequency is still 278 nm, but the emission maximum is shifted to 326 nm, with a 20% lower quantum yield This indicates that only minor changes in tertiary structure have occurred Also, since modification by diethyl pyrocarbonate of the indole nucleus of tryptophan causes complete loss of fluorescence [32] it is evident that neither of the two tryptophan residues per subunit [14] is a target for diethyl pyrocarbonate

Discussion

The rate of inactivation of enoyl-CoA hydratase by diethyl pyrocarbonate is initially rapid, although a second portion of reagent is usually required to obtain complete inactivation The single histidine residue reacts much faster than activity is lost, and

removal of the carbethoxy group from histidine, using hydroxylamine, does not restore activity Evidently histidine plays no role in catalysis The data in Fig 3 suggest that the rapid incorporation of just under one carbethoxy group per subunit has no effect on activity and it is tempting to attribute this incorporation to reaction at histidine Had more radioactive diethyl pyrocarbonate been available, it would have been interesting to test the effect of hydroxylamine treatment on the incorporation of radioactivity into the protein Fig 3 also shows that more residues are subsequently modified by diethyl pyrocarbonate, and this is accompanied by loss of more than 85% of the enzymatic activity Of the four other types of sidechain known to react with diethyl pyrocarbonate [12], tyrosine, cysteine and tryptophan were not modified under the conditions used However, the number of free amino groups per subunit was shown to decrease during modification by diethyl pyrocarbonate The resulting *N*-carbethoxy derivative is known to be acid-stable and resistant to hydroxylamine treatment, as found here

The simplest interpretation of the data in Fig 3 is that, of two residues reacting more slowly with diethyl pyrocarbonate, one is essential for enzymic activity Although the estimates of free amino groups obtained using methyl[1-^{14}C]acetimidate are not very precise, these experiments strongly suggest that these slowly-reacting residues are amino groups Since destruction of the N-terminal serine residue does not abolish enzymic activity, the essential amino group is very probably the ϵ-amino group of a lysine residue

The inactivation of enoyl-CoA hydratase which accompanies modification of cysteine residues [3,7, 8] has been ascribed [8] to alterations in tertiary structure produced by the modification Similarly, inactivation of enoyl-CoA hydratase with the lysine-specific reagents methyl acetimidate [21] or 2,4-pentanedione [33] causes extensive conformational changes, and gradual dissociation of the modified hexameric enzyme into smaller aggregates, probably trimers (Lambiris, S K , unpublished data) With diethyl pyrocarbonate, the results of the sedimentation velocity studies indicate that the hexameric quaternary structure remains intact in the fully modified enzyme. Nevertheless, the possibility that the inactivation is mediated by a conformational change within individual subunits must be considered The fact that the competitive inhibitor acetoacetyl-CoA provides some protection against inactivation suggests, but does not prove, that diethyl pyrocarbonate is indeed modifying a residue at the active site At any rate, a large conformational change is ruled out by the finding that reaction with diethyl pyrocarbonate produces only minor effects on the intrinsic fluorescence of the protein

Work with other hydratases has strongly implicated histidine as an amino acid residue directly involved in the catalytic mechanism Most persuasively, when 3-hydroxydecanoyl-(acyl-carrier-protein) dehydratase is inactivated by the mechanism-based inactivator 2,3-decadienoyl-*N*-acetylcysteamine, a unique histidine residue is covalently labelled [9] Chemical modification of yeast enolase, using diethyl pyrocarbonate, has also shown that histidine residues are apparently essential for activity, and indicated that lysine residues play no role in catalysis or substrate binding [11] In fumarate hydratase, histidine has also been invoked as a direct participant in hydration [10] In contrast, the experiments reported here show that histidine can be eliminated from any role in the catalytic mechanism of enoyl-CoA hydratase Further work will be needed to establish the exact sites of modification by diethyl pyrocarbonate, but these experiments clearly suggest that a single lysine residue is directly involved in catalysis by enoyl-CoA hydratase, and that the unusual *syn* stereochemistry of the hydration [5,6] is not the only mechanistic difference between this enzyme and other hydratases

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