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THE INACTIVATION OF THYMIDYLATE SYNTHASE BY DIETHYL PYROCARBONATE

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Summary

Thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) from *Lactobacillus casei* was inactivated by treatment with diethyl pyrocarbonate. The inactivation was apparently due to the modification of a large proportion of the enzyme's histidine residues. Neither the substrate dUMP nor the product dTMP prevented inactivation by diethyl pyrocarbonate. The inactivated enzyme was not reactivated by treatment with hydroxylamine. These results indicate that histidine residues are involved in the maintenance of enzyme structure.

Introduction

Thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridylate by 5,10-methylene-5,6,7,8-tetrahydrofolate resulting in the production of thymidylate and 7,8-dihydrofolate. The enzyme from an amethopterin-resistant strain of *Lactobacillus casei* has four sulfhydryl groups, one of which is essential for catalytic activity. In the proposed catalytic mechanism, the essential sulfhydryl group acts as a nucleophile [1]; it has been proposed that the nucleophilicity and reactivity of the sulfhydryl group are increased by the proximity of a basic group [2]. A possible candidate for this basic group is a histidine residue. Histidine residues have been shown to increase the reactivity of an essential thiol group in papain [3,4] and succinyl-CoA synthetase [5].

Abbreviations: dUMP, 2'-deoxyuridylate; dTMP, thymidylate; H_4folate, 5,6,7,8-tetrahydrofolate; Pipes, 1,4-piperazinediethanesulfonic acid.

Since diethyl pyrocarbonate has been reported to be a relatively specific reagent for the modification of histidine residues in proteins [6,7], the inactivation of thymidylate synthase by diethyl pyrocarbonate was investigated.

Materials and Methods

Materials

Thymidylate synthase from amethopterin-resistant *L. casei* was purified in the presence of 10 mM 2-mercaptoethanol following the procedure of Lyon et al. [8]. Purified enzyme preparations had specific activities between 3.1 and 3.5 units/mg when assayed in the presence of 25 mM 2-mercaptoethanol by the usual spectrophotometric procedure [9].

Epimeric (±)-H_4folate was prepared by the catalytic hydrogenation of folic acid in acetic acid [10] and was stored at −80° C as a lyophilized powder under argon in sealed serum bottles [11].

Methylmethane thiosulfonate, diethyl pyrocarbonate, dithiothreitol, dUMP, dTMP and imidazole were purchased from Sigma Chemical Co. Pipes was purchased from Calbiochem and hydroxylamine hydrochloride from Eastman.

Methods

Thymidylate synthase was activated and exogenous 2-mercaptoethanol was removed prior to treatment with either diethyl pyrocarbonate or methylmethane thiosulfonate by procedures previously described [12]. The procedure for determining thymidylate synthase activity spectrophotometrically in the absence of thiols has also been described [12].

Diethyl pyrocarbonate was diluted 1 : 20 (v/v) with cold, absolute ethanol. Appropriate amounts of ethanolic diethyl pyrocarbonate solution and absolute ethanol were added to 0.9 ml 0.05 M potassium phosphate, pH 6.8, to give a total volume of 1 ml at the desired diethyl pyrocarbonate concentration. The inhibition mixture was prepared by adding 1 vol. diethyl pyrocarbonate in buffer solution to 4 vol. dethiolated thymidylate synthase solution. The reaction mixture was incubated in an ice bath (unless otherwise specified) and 20-μl aliquots were removed periodically and assayed for activity. Ethanolic diethyl pyrocarbonate solutions were freshly prepared before each experiment and diethyl pyrocarbonate buffer solutions were prepared immediately before use. All solutions were kept in an ice bath. The concentration of the diethyl pyrocarbonate buffer solution was calculated from the increase in absorbance at 230 nm when a 10—40 μl aliquot was added to 10 mM imidazole in 0.1 M potassium phosphate, pH 6.8, using a molar absorptivity coefficient of 3000 $M^{-1} \cdot cm^{-1}$ for the product [6].

Spectra were obtained with a Gilford Model 250 recording spectrophotometer.

Results

Thymidylate synthase from *L. casei* was inactivated by diethyl pyrocarbonate (Fig. 1). The linear plots in Fig. 1A, show that the inactivation reaction is first-order with respect to enzyme concentrations and that the rate is a func-

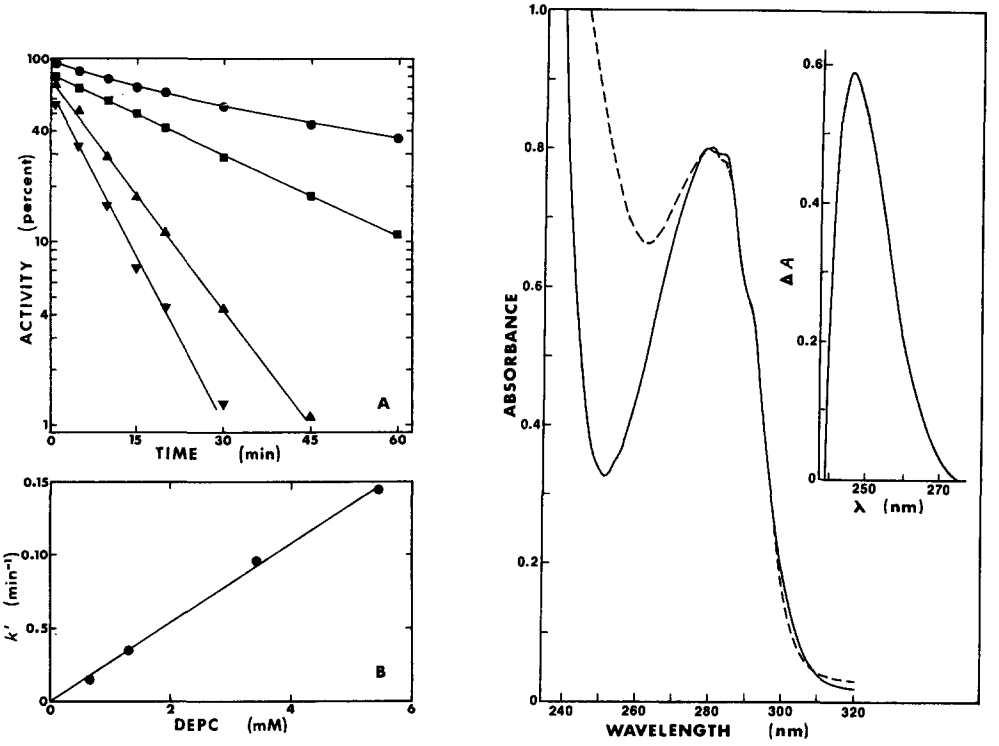
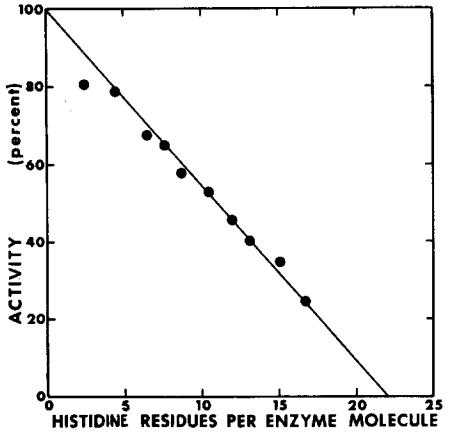


Fig. 1. Inactivation of thymidylate synthase by diethyl pyrocarbonate. A. Activities are plotted on a log scale and are residual activities expressed as percents of the enzymatic activity in reaction mixtures lacking diethyl pyrocarbonate. The enzyme concentration was $8.2 \cdot 10^{-3}$ mM and the diethyl pyrocarbonate concentrations were 0.68 (●——●), 1.32 (■——■), 3.43 (▲——▲) and 5.43 (▼——▼) mM. B. Replot of the apparent first-order rate constants, obtained from the slopes of the lines in Fig. 1A vs. the diethyl pyrocarbonate concentration. DEPC, diethyl pyrocarbonate.

Fig. 2. Spectra of thymidylate synthase in the presence and absence of diethyl pyrocarbonate. The enzyme solutions contained $7.6 \cdot 10^{-3}$ mM thymidylate synthase/0.05 M potassium phosphate, pH 6.8/ 0.4 M potassium chloride/2% ethanol and either with (- - - - - -) or without (———) 3.66 mM diethyl pyrocarbonate. The solution was incubated at 10° C for approx. 30 min. The inset shows the difference spectrum.

tion of the diethyl pyrocarbonate concentration. The straight line obtained (Fig. 1B) when the apparent first-order rate constants, which were determined from the slopes of the lines in Fig. 1A, were plotted against the corresponding concentrations of diethyl pyrocarbonate indicates that the reaction is also first-order with respect to diethyl pyrocarbonate concentration. The apparent second-order rate constant, calculated from the slope of the line in Fig. 1B, is 26 $M^{-1} \cdot min^{-1}$ at 0° C.

A comparison of the spectra of native and diethyl pyrocarbonate-inactivated thymidylate synthase (Fig. 2) shows a difference spectrum with a maximum near 242 nm, which is characteristic of *N*-carbethoxy-histidine residues in proteins [7]. The absence of any appreciable difference in the spectra around 280 nm indicates that diethyl pyrocarbonate did not react with tyrosine residues [13,14]. The number of histidine residues that have reacted can be calculated from the change in absorbance at 242 nm using $\epsilon_{242} = 3200\ M^{-1} \cdot cm^{-1}$ [7]. A



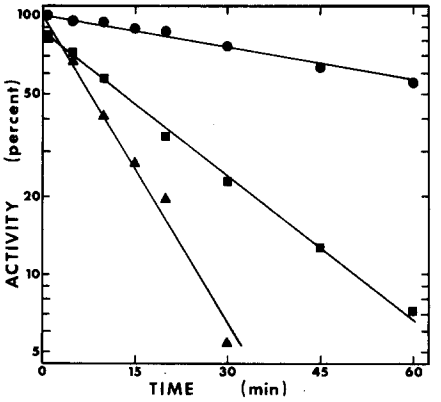


Fig. 3. Relationship of thymidylate synthase activity to the number of histidine residues modified by diethyl pyrocarbonate. The reaction mixture contained $8.2 \cdot 10^{-3}$ mM thymidylate synthase/0.05 M potassium phosphate, pH 6.8/0.4 M KCl/2% ethanol/1.36 mM diethyl pyrocarobonate. The reaction was conducted at 10° C and the absorbance at 242 nm was continuously measured against a similar reaction mixture that lacked diethyl pyrocarbonate. 20-µl aliquots were withdrawn at intervals and assayed for enzymatic activity.

Fig. 4. Protection of thymidylate synthase against inactivation by methylmethane thiosulfonate by dUMP and dTMP. Activities are expressed as percents of the enzymatic activity in reaction mixtures lacking methylmethane thiosulfonate. The concentration of dethiolated enzyme was $8.0 \cdot 10^{-3}$ mM and the methylmethane thiosulfonate concentration was 0.5 mM. dUMP and dTMP concentrations were: 10 mM dUMP (●——●), 10 mM dTMP (■——■) and 1 mM dUMP (▲——▲). Reactions were conducted at 0° C. 20-µl aliquots were removed at the times shown and assayed for enzymatic activity.

plot of the residual activity vs. the number of histidine residues modified (Fig. 3) was linear throughout the loss of the first 75% of the enzymatic activity. Extrapolation of the line to the abscissa shows that complete loss of activity is coincident with the modification of a rather large number of the histidine residues (22 residues of a total of 39 [15]). This does not necessarily mean that all of these histidine residues must be modified for complete inactivation, but it does indicate that the histidine residues, whose modification results in inactivation, react with diethyl pyrocarbonate at the same rate as a large number of total histidine residues.

The inactivation of thymidylate synthase by diethyl pyrocarbonate could not be prevented or retarded by adding either the substrate dUMP or the product dTMP to the incubation mixture. The apparent first-order rate constants with 3.6 mM diethyl pyrocarbonate were 0.11, 0.12 and 0.13 min^{-1} with no additions, 10 mM dUMP and 10 mM dTMP, respectively. Data in Fig. 4, show that these concentrations of nucleotides provided appreciable protection against inactivation by the sulfhydryl reagent methylmethane thiosulfonate [16]. At 0.5 mM methylmethane thiosulfonate, the enzyme lost over 98% activity within 1 min in the absence of a protecting nucleotide. No protection was conferred when dUMP was added 2 min after methylmethane thiosulfonate.

Thymidylate synthase that had been inactivated by diethyl pyrocarbonate was not reactivated by exposure to either 50 or 100 mM hydroxylamine for periods up to 1 h at 30° C. Attempts to reactivate thymidylate synthase with

1 M hydroxylamine resulted in precipitation of the enzyme. No precipitation occurred unless the enzyme had previously been treated with diethyl pyrocarbonate. Thymidylate synthase inactivated by methylmethane thiosulfonate was not reactivated by either 50 or 100 mM hydroxylamine, but full activity could be restored by treatment with 20 mM dithiothreitol for 15 min at 30° C. Dithiothreitol did not restore activity to diethyl pyrocarbonate-inactivated enzyme whether the enzyme had received prior treatment with methylmethane thiosulfonate or not.

Diethyl pyrocarbonate apparently did not cause dissociation of thymidylate synthase into subunits, since there was no appreciable difference in the elution volumes of diethyl pyrocarbonate-treated and untreated enzyme when chromatographed on a column of Sephadex G-100.

Discussion

The data presented show that the inactivation of thymidylate synthase from *L. casei* by diethyl pyrocarbonate is an apparent second-order process. The loss of activity is associated with the modification of a large proportion of the total histidine residues in the enzyme. Unlike some enzymes whose activity can be restored by treatment with hydroxylamine [5,17,18], thymidylate synthase that had been inactivated by diethyl pyrocarbonate was not reactivated by this compound. There must be some irreversible process associated with the inactivation by diethyl pyrocarbonate. The precipitation of only the diethyl pyrocarbonate-treated enzyme that occurs in high concentrations of hydroxylamine also supports this conclusion.

The spectral data clearly show that diethyl pyrocarbonate reacts with histidine residues. There is little, if any, reaction with tyrosine residues as shown by the absence of any appreciable spectral difference in the neighborhood of 280 nm. It is unlikely that diethyl pyrocarbonate reacts with the essential sulfhydryl group, since blocking this group by treatment with methylmethane thiolsulfonate did not prevent the subsequent irreversible inactivation by diethyl pyrocarbonate. The loss of catalytic activity could be partially due to a reaction with catalytically essential groups, but that does not explain either the failure of dUMP or dTMP to protect the enzyme against inactivation or the inability to reactivate the enzyme with hydroxylamine. The data presented neither support nor exclude a possible catalytic role for histidine residues, but they clearly show that histidine residues play an essential role in the maintenance of enzyme structure.

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