Modification of uridine phosphorylase from *Escherichia coli* by diethyl pyrocarbonate

Evidence for a histidine residue in the active site of the enzyme

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Uridine phosphorylase from *Escherichia coli* is inactivated by diethyl pyrocarbonate at pH 7.1 and 10 °C with a second-order rate constant of $840 \text{ M}^{-1} \cdot \text{min}^{-1}$. The rate of inactivation increases with pH, suggesting participation of an amino acid residue with pK 6.6. Hydroxylamine added to the inactivated enzyme restores the activity. Three histidine residues per enzyme subunit are modified by diethyl pyrocarbonate. Kinetic and statistical analyses of the residual enzymic activity, as well as the number of modified histidine residues, indicate that, among the three modifiable residues, only one is essential for enzyme activity. The reactivity of this histidine residue exceeded 10-fold the reactivity of the other two residues. Uridine, though at high concentration, protects the enzyme against inactivation and the very reactive histidine residue against modification. Thus it may be concluded that uridine phosphorylase contains only one histidine residue in each of its six subunits that is essential for enzyme activity.

INTRODUCTION

Uridine phosphorylase (EC 2.4.2.3) catalyses reversible phosphorolysis of uridine and of a number of naturally occurring pyrimidine nucleosides [1–4]. This enzyme also degrades several pyrimidine analogues, e.g. 5-fluorouridine and 5-fluoro-2'-deoxyuridine used in chemotherapy, thus decreasing their therapeutic effectiveness [5–7]. Therefore a great deal of interest is centred on finding inhibitors of uridine phosphorylase that could enhance the therapeutic efficacy of pyrimidine nucleoside analogues [8–13].

The results of studies of the structure–activity relationship for binding of inhibitors to the enzyme [9] support the previous suggestion that the molecule of uridine phosphorylase contains a hydrophobic region. This region seems to be situated adjacent to the binding site of the 5-position of uracil [14,15].

Kinetic studies on the mechanism of catalysis suggested the occurrence of a rapid-equilibrium random mechanism for the action of uridine phosphorylase from *Escherichia coli*, with the formation of an enzyme–uracil–phosphate abortive complex [16].

Crystallographic studies on the enzyme showed that it consists of six identical subunits. The native enzyme has an M_r of 160 000–165 000 and the subunit is of M_r 27 500 [17].

The nature of the active centre has not so far been investigated. The present results show that uridine phosphorylase is modified by diethyl pyrocarbonate, a reagent known to be highly selective for histidyl residues [18,19], and demonstrate that in each subunit of the enzyme only one of the three modifiable histidine residues is essential for enzymic activity.

EXPERIMENTAL

Materials

Diethyl pyrocarbonate, Pipes (disodium salt), uridine and Tris were obtained from Sigma Chemical Co.

Preparation of uridine phosphorylase

Uridine phosphorylase from *E. coli* with a specific activity for uridine phosphorolysis of $240 \mu\text{mol/min per mg}$ was used [20].

The homogeneous enzyme (as checked by polyacrylamide-gel electrophoresis) was obtained by chromatography on a Matrex Gel Green A column, as described by Vita & Magni [21]. The final preparation was subjected to exhaustive dialysis against 50 mM-Pipes buffer, pH 7.1, after which it was stored at -20°C until use.

Enzyme activity assay

The uridine phosphorylase activity was measured in 50 mM-phosphate buffer, pH 7.5. Phosphorolysis of uridine ($280 \mu\text{M}$) by the enzyme was monitored spectrophotometrically by recording at 22°C the decrease in A_{280} with a Uvicon 860 spectrophotometer equipped with a software package applied for kinetic analysis and statistical treatment of the readings. Initial reaction rates were employed in calculations. For conversion of uridine into uracil, $\Delta\epsilon_{280} = 2.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used.

Inactivation of uridine phosphorylase with diethyl pyrocarbonate

The concentration of diethyl pyrocarbonate diluted with acetonitrile was determined spectrophotometrically by the reaction with imidazole at pH 7.5 [22]. The dilute solutions were stable for several weeks.

The first-order rate constant for the decomposition of diethyl pyrocarbonate (k') was calculated from the results obtained by measuring the time-dependent decomposition of the reagent in the modification medium. Samples of the reaction mixture (diethyl pyrocarbonate in 50 mM-Pipes buffer, pH 7.1) were withdrawn at various time intervals, and the amount of non-decomposed diethyl pyrocarbonate was determined by using 15 mM-imidazole [22].

Uridine phosphorylase was inactivated with diethyl pyrocarbonate diluted with acetonitrile. The acetonitrile concentration in the reaction mixture never exceeded 5%. The enzyme ($100 \mu\text{l}$, in 50 mM-Pipes buffer, pH 7.1) was incubated with $5 \mu\text{l}$ of appropriately diluted diethyl pyrocarbonate. To the reference sample $5 \mu\text{l}$ of acetonitrile was added. At various time intervals, samples ($15 \mu\text{l}$) were withdrawn from the incubation mixture and transferred to $20 \mu\text{l}$ of 25 mM-imidazole, to quench the modification reaction. The remaining phosphorolytic activity was determined spectrophotometrically as described above. The

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enzymic activity at each stage of enzyme inactivation was expressed as a fraction of initial activity (A/A_0). The presence of acetonitrile, imidazole and ethoxycarbonylated imidazole did not influence the enzymic activity.

Ethoxycarbonylation of histidine residues

The extent of ethoxycarbonylation of the histidine residues in the enzyme was monitored spectrophotometrically by measuring the time-dependent increase in A_{242} against a blank containing the buffer and an identical concentration of diethyl pyrocarbonate. To calculate the concentration of ethoxycarbonylated histidine residues, $\Delta\epsilon_{242} = 3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used [18].

Hydroxylamine treatment of inactivated enzyme

To study the reversibility of enzyme inactivation by hydroxylamine, uridine phosphorylase in Pipes buffer, pH 7.1, was first treated with diethyl pyrocarbonate until total inactivation. Then imidazole (10 mM) was added to bind the excess of diethyl pyrocarbonate, whereupon the sample was extensively dialysed. Subsequently, hydroxylamine was added to a final concentration of 0.22 M, and the restored enzymic activity was measured at various time intervals. The control sample contained acetonitrile instead of diethyl pyrocarbonate.

Protein determination

Protein was assayed by the method of Bradford [23], with bovine serum albumin as standard.

RESULTS

Inactivation of uridine phosphorylase

Diethyl pyrocarbonate was used to modify histidine residues in uridine phosphorylase. At room temperature the inactivation of the enzyme proceeded at a very high rate. Therefore, to monitor the time-dependent fall in enzymic activity, the reaction was carried out at 10 °C. The fraction of activity remaining (A/A_0), corrected for decomposition of diethyl pyrocarbonate in Pipes buffer, pH 7.1, can be expressed by the equation:

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

Fig. 1. Inactivation of uridine phosphorylase by diethyl pyrocarbonate

Uridine phosphorylase (7 μM) was incubated without (O) or with 0.27 (○), 0.45 (●), 0.60 (△), 0.72 (◇), or 0.95 (◆) mM-diethyl pyrocarbonate in 50 mM-Pipes buffer, pH 7.1, at 10 °C.

Fig. 2. Inactivation of uridine phosphorylase by diethyl pyrocarbonate and re-activation by hydroxylamine

The enzyme (5 μM) was incubated with 0.39 mM-diethyl pyrocarbonate in Pipes buffer, pH 7.1, at 22 °C. After inactivation the sample was treated as described in the Experimental section. k' for decomposition of diethyl pyrocarbonate at 22 °C was estimated to be $38.5 \times 10^{-3} \text{ min}^{-1}$.

where I_0 is the initial concentration of diethyl pyrocarbonate, k is the second-order rate constant for the reaction of the enzyme with the modifier and k' is the first-order rate constant for hydrolysis of the reagent. In Pipes buffer at pH 7.1 and 10 °C k' was estimated to be $19 \times 10^{-2} \text{ min}^{-1}$.

The results obtained from the enzyme-inactivation experiments at various concentrations of diethyl pyrocarbonate are presented as a plot of $\log A/A_0$ versus time $(1 - e^{-k't})/k'$ (Fig. 1). The linearity of these plots indicates that under the conditions used the process approximates to first-order kinetics at all diethyl pyrocarbonate concentrations used.

The relationship between the observed first-order rate constants (k_{obs}) and the concentrations of diethyl pyrocarbonate (DEP) may be described by the equation:

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

where n is the reaction order with respect to diethyl pyrocarbonate concentration, k_{obs} is the pseudo-first-order rate constant, and k is the second-order rate constant with respect to diethyl pyrocarbonate concentration [24].

The second-order rate constant for uridine phosphorylase inactivation at pH 7.1 and 10 °C was calculated to be $840 \text{ M}^{-1} \cdot \text{min}^{-1}$. The linearity of this plot (not shown) indicated that under the inactivation conditions used no reversible diethyl pyrocarbonate–enzyme complex was formed before inactivation [25]. The reaction order, n , of the modifier was calculated to be 0.98, indicating that the molecularity of the inactivation reaction is equal to 1.0.

Reversibility of enzyme inactivation

Treatment of uridine phosphorylase, inactivated by diethyl pyrocarbonate, with hydroxylamine (pH 7.0) restored the activity of the enzyme. The inactivation process was very fast at a relatively low concentration of the modifier, whereas enzyme re-activation proceeded somewhat more slowly but was complete (Fig. 2).

Restoration of enzymic activity with hydroxylamine indicates the modification of either the histidine or tyrosine residues, but not that of the lysine residues or of the thiol groups [22]. Moreover, the reversibility of inactivation indicates that no excess of modifier was used [18] and that the inactivation did not result from denaturation of the enzyme.

Table 1. Effect of uridine concentration on uridine phosphorylase inactivation

Uridine phosphorylase (about 3.8 μM) in 50 mM-Pipes, pH 7.1, was preincubated with uridine for 30 min at room temperature. Then diethyl pyrocarbonate (0.6 mM) was added, and the samples were incubated at the same temperature for 5 min. The reaction was quenched by addition of imidazole (10 mM). The residual activity is expressed as a percentage of that of the blank.

Uridine	Activity remaining (%)
None	5
2 mM	50
5 mM	79
10 mM	85
14 mM	91
20 mM	96

Table 2. Unmodified histidine residues in diethyl pyrocarbonate-treated uridine phosphorylase

Uridine phosphorylase (5.8 μM) in Pipes buffer, pH 7.1, was modified with 0.95 mM-diethyl pyrocarbonate (DEP) at 10 °C. The reaction was quenched by addition of 14 mM-imidazole. Expt. 1, control with acetonitrile (50 $\mu\text{l/ml}$). Expt. 2, enzyme preincubated with 10 mM-uridine for 10 min and then treated with diethyl pyrocarbonate. Expt. 3, enzyme treated with the modifier until residual activity fell to about 10%, whereupon all samples were exhaustively dialysed to remove low- M_r compounds (imidazole, uridine and modifier-imidazole complex). The remaining unmodified histidine residues were determined spectrophotometrically at 242 nm after diethyl pyrocarbonate treatment. The data are means $\pm$ S.D. of three experiments.

Expt. no.	Composition of reaction mixture	Modification time (min)	No. of unmodified histidine residues per subunit
1	Enzyme + acetonitrile	30	2.95 $\pm$ 0.12
2	Enzyme + uridine + DEP	30	1.14 $\pm$ 0.15
3	Enzyme + DEP	3	2.15 $\pm$ 0.22

Protection of uridine phosphorylase against inactivation

Incubation of uridine phosphorylase with diethyl pyrocarbonate in the presence of uridine slowed down the enzyme inactivation (Table 1). Complete protection occurred, though at very high uridine concentrations, which exceeded greatly the K_m value for uridine (130 μM). At lower concentrations of uridine, the enzyme was protected to a lesser extent. The protective effect of uridine against enzyme modification is evident also from the data of experiments presented in Table 2 (Expt. 2). In these experiments, the histidine residue essential for activity (Expt. 3) was protected, but the two non-essential ones were modified. After removal of uridine, subsequent treatment of the enzyme with diethyl pyrocarbonate modified only that one previously protected residue.

Effect of pH on enzyme inactivation

As shown in Fig. 3, the pseudo-first-order rate constant for enzyme inactivation by diethyl pyrocarbonate increases with a rise in pH. The continuous line represents the theoretical curve calculated from the equation [26]:

$$k_{\text{obs.}} = \frac{k_{\text{max.}}}{1 + [\text{H}^+]/K_a} \quad (3)$$

Fig. 3. Effect of pH on enzyme inactivation by diethyl pyrocarbonate

The pH-dependence of the inactivation rate of uridine phosphorylase by 0.214 mM-diethyl pyrocarbonate was determined in 50 mM-Pipes over the pH range 6.0–7.7 at 22 °C. The value of k' for diethyl pyrocarbonate decomposition was determined at each pH. The pseudo-first-order rate constants were calculated from the plots of $\log A/A_0$ versus time. The points in the Figure represent the experimental values, and the continuous curve is calculated with $k = 0.39 \text{ min}^{-1}$ and $k_a = 10^{-6.6}$.

with $k_{\text{max.}} = 0.39 \text{ min}^{-1}$ and $\text{p}K_a = 6.6$ calculated from the intercept and the slope of the plot of $k_{\text{obs.}}[\text{H}^+]$ versus $k_{\text{obs.}}$ respectively. Since diethyl pyrocarbonate reacts only with the unprotonated form of imidazole and also with the imidazole ring of histidine in proteins [27], it may be assumed that the histidine residues in uridine phosphorylase are the candidates for ethoxycarbonylation at pH 7.1.

Modification of histidine residues

The difference spectrum (300–238 nm; not shown) of the diethyl pyrocarbonate-modified uridine phosphorylase versus unmodified enzyme shows only one peak at 242 nm, characteristic of the ethoxycarbonylhistidine residue, and no minimum at 278 nm. These results rule out the possibility of tyrosine modification, and indicate that the inactivation of the enzyme is related to modification of the histidine residues. Under the conditions described in the legend to Fig. 4, about three histidine residues per subunit of enzyme were modified. Measurements of time-dependent ethoxycarbonylation of histidine residues demonstrated that modification of one histidine residue per subunit of uridine phosphorylase was much faster than that of the other two. The time of modification of this residue was equal to the time of complete enzyme inactivation.

The modification of histidine residues and the loss of enzymic activity are presented as a semilogarithmic plot in Fig. 4. The plot of the fraction of unmodified histidine residues versus time is not linear, indicating that these residues are modified at different rates. This also indicates that the histidine residues are not equally accessible to the modifier. The number of histidine residues modified per enzyme subunit was calculated to be 3 (± 0.1).

Graphical analysis of the modification of histidine residues by the method of Ray & Koshland [28] leads to the equation:

$$x = \frac{n-m}{n} = 0.31e^{-0.89t} + 0.69e^{-0.09t} \quad (4)$$

Fig. 4. Loss of activity and content of modified histidine residues in uridine phosphorylase treated with diethyl pyrocarbonate

The enzyme ($7 \mu\text{M}$) was incubated with 0.95 mM -diethyl pyrocarbonate in 50 mM -Pipes buffer, pH 7.1, at 15°C . Enzymic inactivation and modification of the histidine residues were monitored spectrophotometrically (see the Experimental section). The fraction of unmodified histidine residues ($\bullet$) was calculated by assuming the total number of three modifiable residues per subunit as unity. Enzymic activity was expressed as a fraction of the remaining activity, A/A_0 ($\circ$). The fraction of histidine residues modified at a fast rate was obtained by subtracting the contribution of the slowly modified fraction (---) from the total fraction of unmodified histidine residues ($\bullet$) and re-plotting the differences ($\times$).

where x is the total fraction of unmodified residues remaining after the reaction, m is the number of residues modified at time t , and n is the total number of modifiable residues. The plot shown in Fig. 4, calculated from this equation, fits the experimental results well. The values of 0.31 and 0.69, multiplied by the total number, $n = 3$, of modifiable histidine residues in uridine phosphorylase, indicate that the enzyme contains one highly reactive histidine residue per subunit of the enzyme, which is modified with a first-order rate constant $k_1 = 0.89 \text{ min}^{-1}$. Two other histidine residues are modified at a 10-fold lower rate, as seen from the exponential terms representing the respective pseudo-first-order rate constants of modification. Under the same conditions of the experiments, the first-order rate constant for enzyme inactivation, k_a , is 0.90 min^{-1} . This value virtually equals the rate constant k_1 for modification of the fast-reacting histidine residue and suggests that inactivation of uridine phosphorylase by diethyl pyrocarbonate results from the modification of only one histidine residue per subunit.

The data of Table 2, obtained by a distinctly different experimental approach, let us draw similar conclusions to that above. They show that: (i) on average, three histidine residues are modified per enzyme subunit; (ii) uridine protects one histidine residue against modification, but two of three residues are modified within the time of incubation; (iii) within the time of enzyme inactivation, only one histidine residue is modified. Two others remained unmodified.

DISCUSSION

Diethyl pyrocarbonate is known to be a highly selective agent modifying histidine residues in a number of proteins [18,19].

Nevertheless, its reactions with other amino acids have also been reported [18].

Uridine phosphorylase from *E. coli* is rapidly inactivated by diethyl pyrocarbonate. The assumption that histidine-residue modification is responsible for enzyme inactivation is supported by our experimentally determined value of $\text{p}K_a = 6.6$. This value can be assigned to the histidine residue, as it resembles the value characteristic of the modified histidine residues in other proteins [18,19,27,29]. Moreover, the difference spectrum of modified versus unmodified enzyme clearly indicates that the histidine residues, but not those of tyrosine, are modified by diethyl pyrocarbonate.

The reversibility of enzyme inactivation by hydroxylamine indicates that the inactivation resulted from ethoxycarbonylation of the histidine residues and that no excess of the reagent was available for the reaction with the imidazole ring of histidine.

Inactivation of uridine phosphorylase proceeded with first-order kinetics with respect to modifier, whereas ethoxycarbonylation of the histidine residues proceeded at two different rates. The total number of histidine residues accessible to the modifier was found to be three per subunit of the enzyme.

The present results were analysed graphically by the method of Ray & Koshland [28] and statistically (not shown) by the method of Tsou [30]. Both analyses consistently showed that, of the three modifiable histidine residues per subunit, one is essential for activity.

The fast-reacting histidine residue is protected by uridine against modification, which results also in protection of the enzyme against inactivation.

Our experiments represent the first attempt to study the active centre of uridine phosphorylase from *E. coli*. Further investigations are required to establish whether the histidine residue that is essential for the enzyme activity is directly involved in binding of the substrate, or whether it is involved in catalysis.

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REFERENCES

1. Krenitsky, T. A., Barclay, M. & Jacquez, J. A. (1964) *J. Biol. Chem.* **239**, 805-812
2. Pontis, H., Degerstedt, G. & Reichard, P. (1961) *Biochim. Biophys. Acta* **51**, 138-147
3. Birnie, G. D., Kroeger, H. & Heidelberger, C. (1963) *Biochemistry* **2**, 566-572
4. Leer, J. C., Hammer-Jespersen, K. & Schwartz, M. (1977) *Eur. J. Biochem.* **75**, 217-224
5. Heidelberger, C., Greisbach, L., Cruz, O., Schnitzer, R. J. & Grunberg, E. (1958) *Proc. Soc. Exp. Biol. Med.* **97**, 470-475
6. Mukherjee, K. L., Boohar, J., Wentland, D., Ansfield, F. J. & Heidelberger, C. (1963) *Cancer Res.* **23**, 49-66
7. Heidelberger, C. (1975) in *Antineoplastic and Immunosuppressive Agents* (Sartorelli, A. C. & Johns, D. G., eds.), Part II, pp. 193-231, Springer-Verlag, Berlin
8. Niedzwicki, J. G., Chu, S. H., el Kouni, M. H., Rowe, E. C. & Cha, S. (1982) *Biochem. Pharmacol.* **31**, 1857-1861
9. Niedzwicki, J. G., Chu, S. M., el Kouni, M. H. & Cha, S. (1983) *Biochem. Pharmacol.* **32**, 399-415
10. Chu, S. H., Chen, Z. H., Rowe, E. C., Naguib, N. M., el Kouni, M. H. & Chu, Y. M. (1984) *Nucleosides Nucleotides* **3**, 303-311
11. Lin, T. S. & Liu, M. C. (1985) *J. Med. Chem.* **28**, 971-973
12. Veres, Z., Szabolcs, A., Szinai, I., Denes, G., Kajtar-Peredy, M. & Ottvös, L. (1985) *Biochem. Pharmacol.* **34**, 1737-1740
13. Drabikowska, A. K., Lissowska, L., Veres, Z. & Shugar, D. (1987) *Biochem. Pharmacol.* **23**, 4125-4128
14. Baker, B. R. & Kelley, J. L. (1970) *J. Med. Chem.* **13**, 461-467
15. Niedzwicki, J. G., el Kouni, M. H., Chu, S. H. & Cha, S. (1981) *Biochem. Pharmacol.* **30**, 2097-2101

16. Vita, A., Huang, C. Y. & Magni, G. (1983) *Arch. Biochem. Biophys.* **226**, 687–692
17. Cook, W. J., Koszalka, G. W., Hall, W. W., Narayana, S. V. L. & Ealick, S. E. (1987) *J. Biol. Chem.* **262**, 2852–2853
18. Miles, E. W. (1977) *Methods Enzymol.* **47**, 431–442
19. Lundblad, R. L. & Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, vol. 1, pp. 105–125, CRC Press, West Palm Beach, FL.
20. Drabikowska, A. K., Lissowska, L., Draminski, M., Zgit-Wróblewska, A. & Shugar, D. (1987) *Z. Naturforsch.* **42**, 288–296
21. Vita, A. & Magni, G. (1983) *Anal. Biochem.* **133**, 153–156
22. Melchior, W. B., Jr. & Fahrney, D. (1970) *Biochemistry* **9**, 251–258
23. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
24. Levy, H. M., Leber, P. D. & Ryan, E. M. (1963) *J. Biol. Chem.* **238**, 3654–3659
25. Church, F. C., Lundblad, R. L. & Noyes, C. M. (1985) *J. Biol. Chem.* **260**, 4936–4940
26. Burstein, Y., Walsh, K. A. & Neurath, H. (1974) *Biochemistry* **13**, 205–210
27. Holbrook, J. J. & Ingram, V. A. (1973) *Biochem. J.* **131**, 729–738
28. Ray, W. J. & Koshland, D. E., Jr. (1961) *J. Biol. Chem.* **236**, 1973–1979
29. Cousineau, J. & Meighen, E. (1976) *Biochemistry* **15**, 4992–5000
30. Tsou, C. L. (1962) *Sci. Sin.* **11**, 1535–1558

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