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Inactivation of Dihydrofolate Reductase from *Lactobacillus casei* by Diethyl Pyrocarbonate†

Harlow H. Daron* and John L. Aull

ABSTRACT: The role of histidine residues of dihydrofolate reductase from *Lactobacillus casei* was investigated with diethyl pyrocarbonate. This enzyme has no cysteine residues and differs in this respect from many nicotinamide nucleotide dehydrogenases, which have catalytically important sulfhydryl groups. X-ray studies of this enzyme have shown that histidine residues are involved in substrate binding but not in proton transfer [Matthews et al. (1978) *J. Biol. Chem. 253*, 6946]. Dihydrofolate reductase was inactivated by diethyl pyrocarbonate; the second-order rate constant for the reaction was 29 M^{-1} min^{-1} at 0 °C. The difference spectrum of native and diethyl pyrocarbonate inactivated enzyme had a maximum near 242 nm, which indicated a reaction with histidine residues. The absence of any spectral difference near 280 nm indicated that diethyl pyrocarbonate had not reacted with tyrosine residues. Dihydrofolate reductase lost all of its enzymatic activity after about six of the seven histidine residues had been modified. No catalytic activity was lost during an initial rapid reaction with about four histidine residues, but a subsequent slower reaction involving an additional one or two residues was associated with the loss of activity. The enzyme was protected from inactivation by either of the substrates NADPH or dihydrofolate. In fact, treatment with diethyl pyrocarbonate in the presence of either substrate, but particularly with NADPH, resulted in substantially greater activity than that found with untreated enzyme. Treatment with 1 M hydroxylamine partially restored activity to dihydrofolate reductase that had been inactivated by diethyl pyrocarbonate.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the reduction of 7,8-dihydrofolate by NADPH to yield 5,6,7,8-tetrahydrofolate, an essential cofactor in the biosynthesis of thymidylate and purines. Antifolate compounds such as methotrexate, aminopterin, pyrimethamine, and trimethoprim form stable complexes with dihydrofolate reductase and presumably owe their chemotherapeutic activities to the coincident inhibition that ultimately results in decreased DNA biosynthesis.

X-ray studies have shown that dihydrofolate reductases from both *Lactobacillus casei* and *Escherichia coli* have quite similar three-dimensional structures (Matthews et al., 1977, 1978). However, their amino acid sequences are different, though some regions have a high degree of homology (Stone et al., 1977; Freisheim et al., 1978). Many nicotinamide nucleotide dehydrogenases have catalytically essential sulfhydryl groups; a sulfhydryl group in the dihydrofolate reductase from *E. coli* is essential in maintaining an active conformation (Williams & Bennett, 1977), whereas the enzyme from *L. casei* has no cysteine residues. Histidine residues have been shown to be catalytically essential in a number of nicotinamide nucleotide dehydrogenases, where their proposed function is as a proton donor and acceptor (Dalziel, 1975). No histidine residue is involved in proton transfer in dihydrofolate reductase from either *L. casei* or *E. coli* (Matthews et al., 1978), but histidine residues are involved in substrate binding to the *L. casei* enzyme (Birdsall et al., 1977; Matthews et al., 1978, 1979) and in maintaining an active conformation of the *E. coli* enzyme (Greenfield, 1974; Williams, 1975). Two histidine residues of dihydrofolate reductase of *E. coli* react rapidly with diethyl pyrocarbonate, resulting in a loss in catalytic activity (Greenfield, 1974). There is little if any correspondence between the position of the seven histidines in the amino acid sequence of the *L. casei* enzyme and the five histidines in the *E. coli* enzyme (Freisheim et al., 1978). Despite the structural similarities between the *E. coli* and *L. casei* enzymes, they differ in a number of respects that might

† From the Departments of Animal & Dairy Sciences (H.H.D.) and Chemistry (J.L.A.), Auburn University, Auburn, Alabama 36849. *Received August 3, 1981.* This research was supported in part by Grant GM 27013 (to J.L.A.) from the National Institutes of Health. Alabama Agricultural Experiment Station Journal Series No. 4-810079.

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be related to the details of the catalytic mechanism, and so it was of interest to investigate the effect of modifying histidine residues with diethyl pyrocarbonate on the activity of dihydrofolate reductase from a methotrexate-resistant strain of *L. casei*. A preliminary report of this study has been previously presented (Aull & Daron, 1981).

Experimental Procedures

L-7,8-Dihydrofolic acid was prepared by reducing L-folic acid with sodium dithionite (Blakley, 1960). NADPH and diethyl pyrocarbonate were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Enzyme Purification and Assay. Dihydrofolate reductase was purified from a methotrexate-resistant strain of *L. casei* by the method of Liu & Dunlap (1974). The purified enyzme was homogeneous by analytical polyacrylamide gel electrophoresis of both the native enzyme and the NADPH–enzyme binary complex formed by incubating the enzyme with a 2-fold molar excess of NADPH (Dunlap et al., 1971). Enzymatic activity was determined with a Gilford Model 250 recording spectrophotometer by measuring the decrease in absorbance at 340 nm due to the disappearance of dihydrofolate and NADPH (Liu & Dunlap, 1974). A typical assay mixture contained 0.9 mL of 0.05 M Tris-HCl buffer, pH 7.5, 40 μL of 2.3 mM dihydrofolate, and 40 μL of 3.3 mM NADPH; the reaction was initiated by the addition of 20 μL of enzyme solution. Enzyme concentrations were determined by using an absorptivity coefficient of 26400 M^{-1} cm^{-1} at 278 nm (Liu & Dunlap, 1974).

Inhibition of Dihydrofolate Reductase by Diethyl Pyrocarbonate. Diethyl pyrocarbonate was stored desiccated near 0 °C to minimize decomposition by hydrolysis. Ethanolic diethyl pyrocarbonate solutions were prepared on the day of the experiment by diluting diethyl pyrocarbonate 1:19 (v:v) with cold absolute ethanol. Buffered diethyl pyrocarbonate solutions were prepared immediately before use by adding appropriate amounts of ethanolic diethyl pyrocarbonate solution and absolute ethanol to 0.9 mL of 0.05 M Tris-HCl buffer, pH 7.5, to give a total volume of 1 mL at the desired diethyl pyrocarbonate concentration. Concentrations of the buffered diethyl pyrocarbonate solutions were calculated from the increase in absorbance at 230 nm when an aliquot was added to 10 mM imidazole in 0.1 M potassium phosphate buffer, pH 6.8, by using an absorptivity coefficient of 3000 M^{-1} cm^{-1} for the product (Melchior & Fahrney, 1970). All solutions were kept in an ice bath.

Inhibition mixtures were prepared by adding 1 volume of buffered diethyl pyrocarbonate solution to 4 volumes of dihydrofolate reductase solution. The reaction mixtures were incubated in an ice bath (unless otherwise specified), and 5- or 20-μL aliquots were withdrawn periodically and assayed for activity. Control inhibition mixtures, which lacked diethyl pyrocarbonate, were prepared and treated as described above except the buffered diethyl pyrocarbonate solution was replaced by a solution consisting of 0.1 mL of absolute ethanol and 0.9 mL of buffer. The number of histidine residues that reacted with diethyl pyrocarbonate was calculated from the change in absorbance at 242 nm by using an absorptivity coefficient of 3200 M^{-1} cm^{-1} (Miles, 1978).

Spectra were obtained with a Gilford Model 250 recording spectrophotometer equipped with a Model 2530 wavelength scanner.

Results

Dihydrofolate reductase from *L. casei* lost essentially all of its enzymatic activity when treated with diethyl pyrocarbonate.

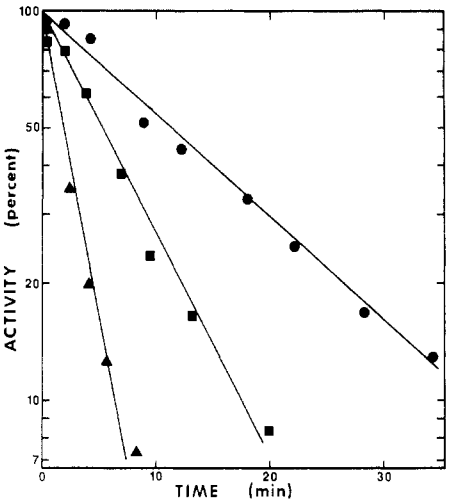

FIGURE 1: Inactivation of dihydrofolate reductase by diethyl pyrocarbonate. Activities are plotted on a logarithmic scale and are residual activities expressed as percents of the enzymatic activity in reaction mixtures lacking diethyl pyrocarbonate. The enzyme concentration was 1.45×10^{-2} mM, and the diethyl pyrocarbonate concentrations were 1.99 (●), 5.67 (■), and 10.15 (▲) mM.

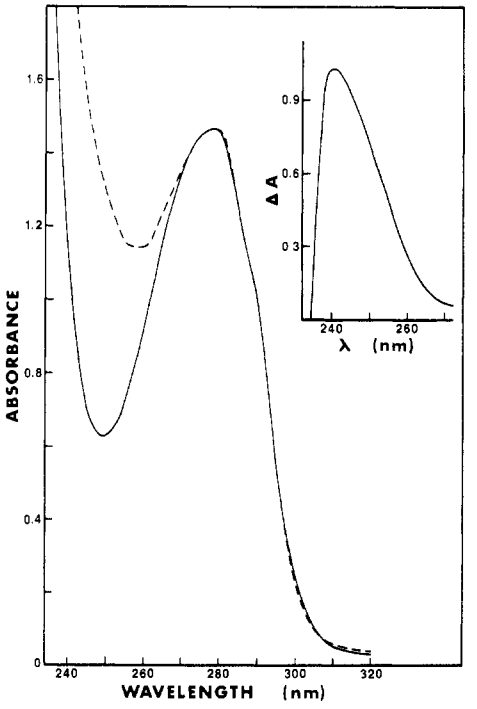

FIGURE 2: Spectra of dihydrofolate reductase in the presence and absence of diethyl pyrocarbonate. The enzyme solutions contained 5.5×10^{-2} mM dihydrofolate reductase, 0.05 M Tris-HCl, pH 7.5, 2% ethanol, and either 3.24 mM (---) or no (—) diethyl pyrocarbonate. The solution was incubated at approximately 15 °C for over 30 min.

Semilogarithmic plots of residual activity vs. time (Figure 1) were linear, indicating that the inactivation reaction was first order with respect to enzyme concentration. The rate of the reaction was a function of the diethyl pyrocarbonate concentration, and a plot of the apparent first-order rate constants, determined from the slopes of the lines in Figure 1, vs. the corresponding concentrations of diethyl pyrocarbonate also gave a straight line. The apparent second-order rate constant for the inactivation reaction, calculated from the slope of that line, was 29 M^{-1} min^{-1} at 0 °C. Control reaction mixtures that lacked diethyl pyrocarbonate lost no enzymatic activity under identical conditions.

The UV absorption spectra of native and diethyl pyrocarbonate inactivated dihydrofolate reductase were different

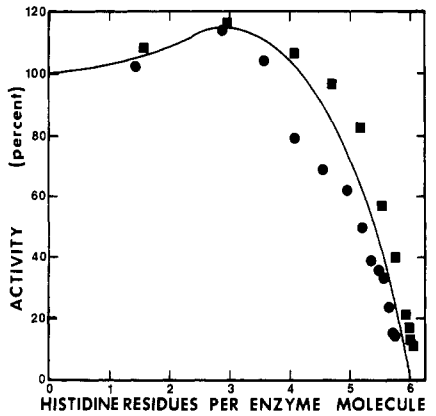

FIGURE 3: Relationship of dihydrofolate reductase activity to the number of histidine residues modified by diethyl pyrocarbonate. The reaction mixtures in two separate experiments contained either 5.52 $\times 10^{-2}$ (●) or 5.38 $\times 10^{-2}$ (■) mM dihydrofolate reductase, 0.05 M Tris-HCl, pH 7.5, 2% ethanol, and 3.24 mM diethyl pyrocarbonate. The reactions were conducted at about 15 °C, and the absorbance at 242 nm was continuously measured. The absorbance was intially set by using a similar reaction mixture that lacked diethyl pyrocarbonate. Five-microliter aliquots were withdrawn at intervals and immediately added to 0.955 mL of ice-cold 0.05 M Tris-HCl, pH 7.5, containing 0.138 mM NADPH to stop further inactivation by diethyl pyrocarbonate. After 30 min, when the reaction was apparently complete, i.e., no further change in A_{242}, the samples that had been withdrawn were warmed to 30 °C and assayed for enzymatic activity as previously described by adding 40 μL of dihydrofolate solution. Neither the enzymatic activity nor A_{242} of control reaction mixtures changed during the reaction period.

(Figure 2). The difference spectrum had a maximum near 242 nm, which is characteristic of *N*-carbethoxyhistidine residues in proteins (Miles, 1978). The absence of any appreciable difference between the spectra in the region of 280 nm indicated that diethyl pyrocarbonate did not react with tyrosine residues (Mühlárd et al., 1967; Burstein et al., 1974). The number of histidine residues that had reacted was calculated from the change in absorbance at 242 nm. Figure 3 is a plot of the residual activity vs. the number of histidine residues that was modified. Results from two separate experiments with slightly different enzyme concentrations are shown. There are seven histidine residues in dihydrofolate reductase from *L. casei* (Freisheim et al., 1978). Data in Figure 3 show that three or four of these residues reacted without any appreciable loss of enzymatic activity. This reaction occurred within the first 2 min of the reaction period. Loss of activity was associated with the subsequent modification of one or two slower-reacting residues. Apparently, one histidine residue failed to react during the 30-min reaction period.

The inactivation of dihydrofolate reductase by diethyl pyrocarbonate was prevented or retarded by including either NADPH or dihydrofolate in the reaction mixture (Table I). The exact degree of protection provided by the substrates was difficult to determine since treatment with diethyl pyrocarbonate in their presence substantially increased the enzymatic activity above that of nontreated controls. What appears to be complete protection was afforded by NADPH at a concentration 46% greater than that of dihydrofolate reductase, while a comparable concentration of dihydrofolate gave only partial protection. These results are in accord with the observations that dihydrofolate reductase forms a very stable binary complex with NADPH ($K_{association} > 10^8$ M^{-1}) and a less stable binary complex with dihydrofolate ($K_{association} = 2.25 \times 10^6$ M^{-1}) (Dann et al., 1976). At higher concentrations both NADPH and dihydrofolate provided apparent full protection, since enzymatic activities greater than those of nontreated

Table I: Substrate Protection of Dihydrofolate Reductase from Inactivation by Diethyl Pyrocarbonate[a]

substance added	concn (μM)	rel act. (% of control) with DEPC	rel act. (% of control) without DEPC
none		0	100[b]
NADPH	6.35	181	115
NADPH	63.5	210	112
H_2folate	6.51	45	107
H_2folate	65.1	155	106

[a] The concentration of dihydrofolate reductase in the reaction mixtures was 4.36 μM, and the concentration of diethyl pyrocarbonate was 6.27 mM when present. Reactions were started by the addition of diethyl pyrocarbonate. The reaction mixtures were incubated at 0 °C, and 20-μL aliquots were removed after 40 min and assayed for enzymatic activity. [b] The activity of the control reaction mixture, which contained no diethyl pyrocarbonate or substrates, was set at 100%.

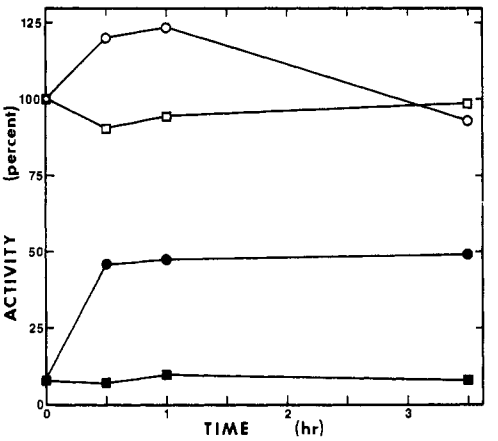

FIGURE 4: Reactivation of diethyl pyrocarbonate inactivated dihydrofolate reductase with hydroxylamine. Dihydrofolate reductase was inactivated by incubating 5.15 $\times 10^{-2}$ mM enzyme at 0 °C for approximately 30 min in the presence (closed symbols) or the absence (open symbols) of 6.9 mM diethyl pyrocarbonate. Assays of these reaction mixtures showed that the treated enzyme had 8% of the activity of the untreated enzyme. Reaction mixtures were then diluted with an equal volume of hydroxylamine solution prepared in 0.05 M Tris-HCl, pH 7.5, and adjusted to pH 7.5 with KOH. Hydroxylamine concentrations in the reactivation mixtures were 1 M (circles) or 0.1 M (squares). Incubation was conducted at approximately 22 °C, and aliquots were removed and assayed for enzymatic activity at the times shown.

controls were obtained. The data also show that in the absence of diethyl pyrocarbonate, dihydrofolate reductase activity was slightly stimulated by NADPH and possibly by dihydrofolate.

Hydroxylamine partially reactivated dihydrofolate reductase that had been inactivated by diethyl pyrocarbonate (Figure 4). Approximately 50% of the initial enzymatic activity was restored by 1 M hydroxylamine within the first 30 min with no further change during the next 3 h. Enzyme that had not been inactivated by diethyl pyrocarbonate showed a modest, transient activation when subjected to the same treatment. A lower concentration of hydroxylamine (0.1 M) did not restore the activity of enzyme that had been inactivated by diethyl pyrocarbonate and had no effect on untreated enzyme. The lack of complete reactivation by hydroxylamine could be due to either an irreversible conformational change or the reaction of diethyl pyrocarbonate with residues other than histidine. The most likely candidate in this respect is lysine. Greenfield (1974) has shown that diethyl pyrocarbonate reacted with some lysine residues of dihydrofolate reductase from *E. coli*

but that these residues were not catalytically important.

Discussion

In the following discussion we attempt to correlate our results with the X-ray data of Matthews et al. (1978, 1979) and the ^{1}H NMR data of Birdsall et al. (1977), Wyeth et al. (1980), Feeney et al. (1980), and Gronenborn et al. (1981). Histidine resonances have been tentatively assigned to specific residues by Matthews (1979) and Wyeth et al. (1980). One should keep in mind, however, that these three studies have been conducted with three independently isolated, methotrexate-resistant strains of *L. casei*. The strain we used was the same as that for which the amino acid sequence has been determined by Freisheim et al. (1978). Matthews et al. (1978, 1979) used this amino acid sequence to interpret their electron density map, and the good agreement led them to conclude that "if any differences do exist they must be very minor". The amino acid sequence (Freisheim et al., 1978) has seven histidine residues at positions 18, 22, 28, 64, 77, 89, and 153. Initially, Birdsall et al. (1977) obtained ^{1}H NMR data for six histidines, which was in accordance with the amino acid composition data previously reported by Dann et al. (1976), but recently the C2 proton resonance of a seventh histidine, with unusual characteristics, was detected far downfield from the other histidine C2 resonances (Wyeth et al., 1980). The reported amino acid sequence for the enzyme from this strain is identical with that given by Freisheim et al. (1978) except for the substitution of Asp for Asn at residue 8 (Morris, 1980).

The loss of catalytic activity is associated with the modification of one or two histidine residues by diethyl pyrocarbonate. We suggest that the most likely candidates are histidine-18, histidine-22, or both, even though these residues have no obvious catalytic function. Both residues are in an extended loop consisting of residues 12–21, which moves 2–3 Å when NADPH is bound (Matthews et al., 1978, 1979). There is general sequence homology in this region for the dihydrofolate reductases of *L. casei*, *E. coli*, and *Streptococcus faecium* (Freisheim et al., 1978), and the sequence containing residues 19–24 is highly conserved (Freisheim et al., 1977). Glycine-17, histidine-18, leucine-19, and tryptophan-21 interact directly with bound NADPH (Matthews et al., 1979). Tryptophan-21 has been implicated in catalysis and is conserved in enzymes from at least five different species (Freisheim et al., 1977). When NADPH binds, tryptophan-21 moves about 3 Å more deeply into the active-site region without altering the conformation of its side chain, so the movement is "exclusively a result of conformationally induced tightening of this portion of the protein backbone loop" (Matthews et al., 1979). Recent studies by Feeney et al. (1980) using photochemically induced dynamic nuclear polarization NMR (photo-CIDNP) provide strong evidence of substrate- and inhibitor-induced conformational changes that involve a histidine residue (A), which they suggest is probably histidine-22. If this movement is an essential feature of the catalytic mechanism, as seems likely, then reaction with diethyl pyrocarbonate might result in loss of enzymatic activity if it prevented the conformational change involving the protein loop containing these histidine residues.

The protection provided by either NADPH or dihydrofolate against inactivation by diethyl pyrocarbonate also suggests that histidine-18 and/or histidine-22 are involved, since these residues are close to the juncture of bound NADPH and bound methotrexate (or, presumably, dihydrofolate) (Matthews et al., 1978). The protection could simply be due to denying diethyl pyrocarbonate access to the histidine residues. Experimental support comes from Feeney et al. (1980), who reported that histidine A (probably histidine-22) is inaccessible in the binary complexes with $NADP^+$ or folate (but, interestingly, becomes accessible when the ternary complex is formed). However, another possibility is that substrate binding induces a catalytically favorable conformation without preventing reaction with diethyl pyrocarbonate. A subsequent reaction with diethyl pyrocarbonate in which the catalytically active conformation is preserved might explain the substantial activation observed when dihydrofolate reductase is treated with diethyl pyrocarbonate in the presence of NADPH. The reaction of diethyl pyrocarbonate with histidine-18 and/or histidine-22 is thus imagined to stabilize whichever conformation the enzyme has and so results in inactivation when substrates are absent or activation when substrates are bound.

Histidines-64 and -77 are involved in NADPH binding, but they bind the adenosine moiety of NADPH and are well removed from the methotrexate (dihydrofolate) binding site (Matthews et al., 1979). If the loss of activity with diethyl pyrocarbonate is due to modification of these residues, it is not clear how dihydrofolate affords protection. It has been shown that histidine-64 remains accessible when folate and folate derivatives are bound to the enzyme (Feeney et al., 1980; Wyeth et al., 1980). The same reasoning and the protection provided by NADPH made it unlikely that inactivation by diethyl pyrocarbonate is due to modification of histidine-28, since it is involved in binding of the glutamate moiety of methotrexate (Matthews et al., 1978) and remains accessible when $NADP^+$ or trimethoprim is bound (Feeney et al., 1980; Wyeth et al., 1980; Gronenborn et al., 1981).

Our data indicate that during the loss of enzymatic activity diethyl pyrocarbonate reacted with six rather than all seven histidine residues. A possible interpretation is that one specific histidine residue did not react, perhaps because it either was inaccessible to diethyl pyrocarbonate or had an exceptionally high pK. The most likely candidate appears to be histidine-153. Matthews (1979) reported that this residue is in an inaccessible environment. A histidine with an abnormally high pK, designated G, has been shown to be inaccessible by deuterium substitution (Wyeth et al., 1980) and photo-CIDNP (Feeney et al., 1980). Histidine G has been assigned to histidine-153 by Matthews (1979), and Wyeth et al. (1980) feel that it could be either histidine-153 or histidine-89. However, histidine-153 is homologous with histidine-149 in dihydrofolate reductase from *E. coli* (Freisheim et al., 1978), which reacts rapidly with diethyl pyrocarbonate (Poe et al., 1979). Poe et al. (1979) found that histidine-124 of dihydrofolate reductase from *E. coli* reacted poorly, if at all, but the homologous region of the enzyme from *L. casei* has no histidine residues. Another possible interpretation is that all histidine residues are potentially reactive and the unreacted residues are randomly selected and are distributed among all, or a restricted group, of the total histidine residues. The selection might be simply on a statistical basis, or prior reaction of one histidine residue with diethyl pyrocarbonate might prevent or retard reaction with another. For example, histidine-18 or histidine-22, but not both, might be carbethoxylated.

A modest increase in enzymatic activity of approximately 10–20% that was unrelated to reaction with diethyl pyrocarbonate was found when the enzyme was incubated with NADPH (Table I) and when the enzyme was treated with 1 M hydroxylamine (Figure 4). Others have reported that this enzyme is also activated by NaCl at pH 7.35 but not at pH 8.7 (Dann et al., 1976) and by urea (Gundersen et al., 1972). Dihydrofolate reductases from a number of sources are similarly stimulated by a variety of reagents that include salts,

urea, thiourea, and guanidinium chloride [e.g., Kaufman (1968, and references cited therein)]. Organic mercurial sulfhydryl reagents activate the enzyme from mammalian, but not bacterial, sources (Kaufman et al., 1980, and references cited therein). The mechanism of activation by these compounds is unknown. Even the slight increase in activity that was observed when about three histidine residues had reacted with diethyl pyrocarbonate (Figure 3) may be related to these other activations.

Acknowledgments

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Subcellular Transport and Ribosomal Incorporation of Microinjected Protein S6 in Oocytes from *Xenopus laevis*†

Holger Kalthoff and Dietmar Richter*

ABSTRACT: Protein S6 was isolated from 40S ribosomal subunits of *Xenopus laevis* oocytes, labeled with sodium boro[3H]hydride, and microinjected back into oocytes. In the first 4 h of incubation, the uptake of S6 into the nucleus increased to a maximum, with, however, no detectable incorporation into 40S ribosomal subunits. After this lag period, S6 was progressively integrated into the small ribosomal subunit. When rRNA transcription was inhibited by actinomycin D, the uptake of S6 into the nucleus and its consequent incorporation into the 40S subunit were significantly reduced. Moreover, when enucleated oocytes were microinjected, little or no S6 was found in the 40S subunits, also suggesting that integration of S6 into ribosomes is linked to rRNA precursor synthesis. In contrast to S6, the acidic protein eL12 isolated from *Artemia salina* or *X. laevis* oocyte 60S subunits was integrated into the large subunit independently of the nucleus or active rRNA synthesis.

The assembly of various ribosomal proteins and rRNAs to a functional ribosome has been investigated in great detail in procaryotes. This process comprises a sequence of steps whereby some proteins are involved at an early and others at a late assembly stage (Nomura & Held, 1974; Nierhaus et al., 1973). Beside the larger number of ribosomal proteins (Wool, 1980), attempts to study this process in eucaryotes are complicated by the fact that the various stages take place in different cellular compartments (Warner et al., 1980); most

† From the Institut für Physiologische Chemie der Universität Hamburg, Abteilung Zellbiochemie, 2000 Hamburg 20, Federal Republic of Germany. *Received July 24, 1981.* This research was financially supported by the Deutsche Forschungsgemeinschaft.

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