Modification of histidine residues by diethyl pyrocarbonate leads to inactivation of the *Rhodospirillum rubrum* RrF_1-ATPase

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The RrF_1-ATPase from the photosynthetic bacterium *Rhodospirillum rubrum* was rapidly and completely inactivated by diethyl pyrocarbonate (DEPC) at pH 6.0 and 22°C. When applied in 1 step 5 mM DEPC were required for >90% inactivation and this DEPC-modified enzyme showed an increase in absorption at 242 nm as well as a decrease in absorption at 280 nm, suggesting modification of both histidine and tyrosine residues. Complete inactivation of the RrF_1-ATPase could be obtained with only 250 μM DEPC when applied in 5 separate steps of 50 μM each. The only absorption change observed under these conditions was an increase at 242 nm indicating that the inactivation can be correlated with modification of histidine residues. Complete inactivation requires the modification of 2–3 histidine residues per molecule of RrF_1.

Bacterial ATPase Rhodospirillum rubrum *Histidine residue* *Diethyl pyrocarbonate* *Inhibition of ATPase*

1. INTRODUCTION

The proton-translocating $F_0 \cdot F_1$-ATP synthase present in energy-transducing membranes is composed of an integral membrane sector, designated F_0, and a peripheral membrane sector, designated F_1. The F_1 sector, that contains the active site for ATP synthesis, can be resolved from these membranes quite easily. But after its solubilization it is only capable of catalyzing the net hydrolysis of ATP and is therefore referred to as the F_1-ATPase (reviews [1,2]). Although F_1-ATPases from a variety of species have been studied in detail, little is known about the structure of their active site. Chemical modification studies have provided valuable information regarding the identification of possible essential amino acid residues [3]. Thus, covalent labeling of a number of F_1-ATPases by reagents known to specifically modify arginine, lysine, tyrosine and carboxyl groups has been found to result in inhibition of their hydrolytic activity. There is, however, no information concerning the involvement of histidine residues in the activity of any F_1-ATPase. Interestingly, in the very few cases where the amino acid sequence around the modified residues has been examined, a histidine residue appeared next to both modified tyrosine [4] and glutamic acid [5,6].

We have reported on the inactivation of the RrF_1-ATPase isolated from the photosynthetic bacterium *R. rubrum* by 4-chloro-7-nitrobenzofurazan and dicychlohexylcarbodiimide [7,8]. These reagents are known to interact, respectively, with tyrosine and carboxyl groups of F_1-ATPases [3]. Here, we have investigated the inactivation of the RrF_1-ATPase by DEPC. This reagent has been shown to inhibit the activity of enzymes by carbethoxylating various amino acid residues, but when applied in aqueous solutions at pH 6.0 it modified

Abbreviations: DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid

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histidine residues in proteins with considerable specificity [9]. In the case of RrF_1-ATPase its incubation with DEPC even at pH 6.0 has resulted in modification of both histidine and tyrosine residues. However, by changing the procedure of incubation with DEPC, conditions were found under which the inactivation of the RrF_1-ATPase could be correlated with a specific modification of histidine residues.

2. EXPERIMENTAL

Rhodospirillum rubrum cells were grown as in [10]. Chromatophores were prepared by the Yeda Press according to [11,12] in the presence of deoxyribonuclease and ribonuclease [13]. The RrF_1-ATPase was solubilized, purified, and stored as in [7]. Before any treatment with DEPC the RrF_1-ATPase was freed from the storage buffer by elution–centrifugation [14] on a Sephadex G-50 column equilibrated with the modification buffer containing 50 mM Mes–NaOH (pH. 6.0), 2 mM EDTA, 50 mM NaCl and 20% glycerol.

Inactivation by DEPC was carried out by incubating the RrF_1-ATPase in the modification buffer with various concentrations of DEPC at 22°C. Stock solutions of 0.2–1.0 M DEPC in absolute ethanol were freshly prepared for each experiment and the final ethanol concentration did not exceed 1% by volume. At various time intervals aliquots of the modification mixture were diluted 200-fold in 50 mM Tricine–NaOH (pH 8.0) and immediately assayed for Ca^{2+}-ATPase activity. Control experiments were run under identical conditions with the same final concentration of ethanol except that DEPC was omitted.

The Ca^{2+}-ATPase activity was assayed for 10 min at 35°C in a final volume of 1 ml containing 50 mM tricine–NaOH (pH 8.0), 4 mM $CaCl_2$ and 5–10 μg enzyme. The reaction was started by the addition of 4 mM ATP. The amount of phosphate released was measured colorimetrically [15]. Protein was determined as in [16]. The difference spectra of DEPC-treated *vs.* untreated enzyme were obtained on the Cary Model 16 spectrophotometer. The kinetics of modification of histidine residues were followed by the differential absorption at 242 nm. The number of modified histidine residues was calculated by using 3200 $M^{-1}.cm^{-1}$ as the molar absorption coefficient for carbethoxyhistidine [17]. M_r 350000 for the RrF_1-ATPase [18] was used in all calculations.

3. RESULTS AND DISCUSSION

Incubation of RrF_1-ATPase with DEPC at pH 6.0 and 22°C resulted in a very rapid loss of enzyme activity, but the semilog plot of residual Ca^{2+}-ATPase activity *vs* time did not yield a straight line (fig. 1). The rate of inactivation decreased practically to zero after about 3 min with all tested DEPC concentrations. This pattern of inhibition by DEPC was reported with other enzymes [19,20]. It was found to be due to instability of DEPC, which is readily hydrolyzed in aqueous solutions, its half-life being dependent on temperature, pH, and the buffer employed [21]. Thus, although in order to maximise the reaction of DEPC with the protein a relatively high concentration of 11.4 μM RrF_1-ATPase was employed (fig. 1), its inactivation by >85% required the application of DEPC at a molar excess of 400. Under these conditions the changes in the absorption spectrum of RrF_1-ATPase during inactivation with DEPC showed a simultaneous increase at 242 nm and decrease at 280 nm (fig. 2). The 242 nm change is indicative of histidine modification [17,22] and the 280 nm change indicates a modification of tyrosine [22]. So in RrF_1-ATPase, unlike

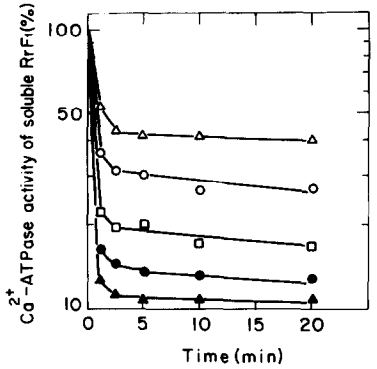

Fig. 1. Kinetics of inactivation of the RrF_1-ATPase by DEPC. RrF_1-ATPase (4 mg/ml) was incubated with 0.2 mM (△), 1.0 mM (○), 2.5 mM (□), 5 mM (●) and 10 mM (▲) DEPC as in section 2. At the indicated time intervals, 5 μl aliquots were diluted 200-fold and assayed. The control Ca^{2+}-ATPase activity was 8.0±0.1 μmol.min.mg of protein^{-1}.

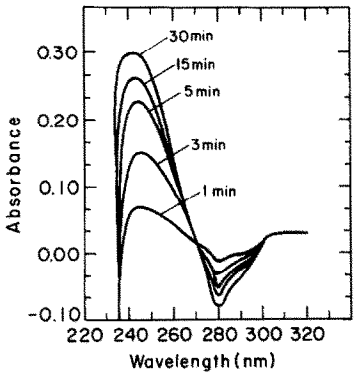

Fig. 2. Ultraviolet difference spectra of RrF_1-ATPase obtained after various periods of inactivation with 5 mM DEPC. The cuvettes contained 1 ml of the enzyme (4 mg/ml) in the modification buffer; 10 μl 0.5 M DEPC in ethanol was added to the sample cuvette, and an equal volume of ethanol was added to the reference cuvette. The difference spectra were recorded after the indicated periods of inactivation.

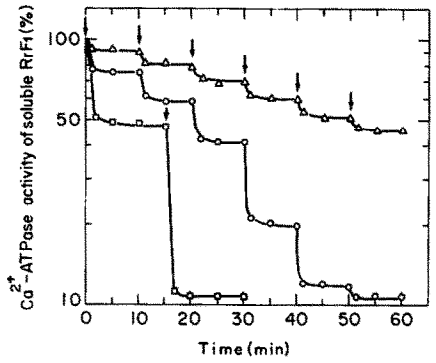

Fig. 3. Inactivation of the RrF_1-ATPase by stepwise addition of DEPC. Conditions were as described in fig. 1, except that at the times indicated by the arrows fresh DEPC was added at 25 μM ($\triangle$), 50 μM (○) and 200 μM (□).

in other enzymes [9], DEPC even at pH 6.0 does not interact specifically with histidine residues but carbethoxylates also tyrosine residues, at least at the 400-fold molar excess required for complete inhibition.

A possible way to differentiate between the modification of histidine and tyrosine residues and establish which of these modifications correlates with the enzyme inactivation is to treat the DEPC-modified enzyme with hydroxylamine, that has been shown to remove the carbethoxy group from modified histidine more readily than from modified tyrosin [9]. In the case of the RrF_1-ATPase this approach was ineffective. We have tried a wide range of hydroxylamine concentrations applied for varying time intervals. But incubation of the RrF_1-ATPase modified by 5 mM DEPC with up to 0.5 M hydroxylamine for 1.5 h did not lead to any reactivation, whereas incubation with only 0.1 M hydroxylamine for 16 h led to complete inhibition of the RrF_1-ATPase by the hydroxylamine itself, despite the fact that before each assay excess hydroxylamine was removed either by elution–centrifugation or by dialysis. So, the 400-fold molar excess of DEPC required for inactivation of RrF_1 leads to carbothoxylation of both histidine an tyrosine, that can be reversed by hydroxylamine. We have therefore searched for possible treatments that would decrease the concentration of DEPC required for the inactivation of RrF_1-ATPase.

An approach that proved fruitfull was to treat the enzyme with stepwise additions of low concentrations of DEPC (fig. 3). Thus, with 200 μM DEPC, which when added once caused 50–60% inhibition (see figs. 2,3), a second addition resulted in 90% inactivation. A total of 250 μM DEPC (which comprises only a 20-fold molar excess of reagent over enzyme) applied 5 consecutive steps of 50 μM each over 50 min led to complete inactivation of the RrF_1-ATPase (fig. 3). The only absorption change observed under these conditions was an increase at 242 nm (fig. 4). So, when in

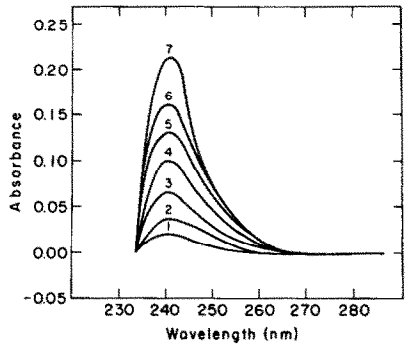

Fig. 4. Ultraviolet difference spectra of RrF_1-ATPase obtained after the indicated number of stepwise additions of 50 μM DEPC. Conditions were as described in fig. 2. Each difference spectrum was recorded 10 min after the addition of DEPC.

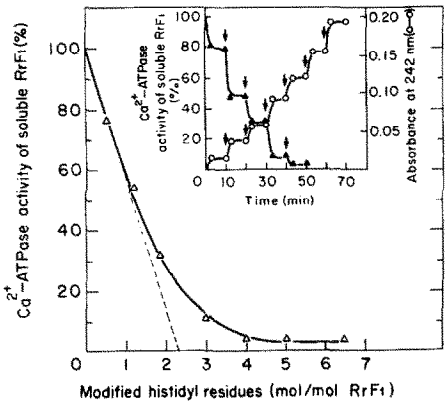

Fig. 5. Correlation between inactivation of the RrF_1-ATPase and the modification of histidine residues as measured by the difference spectra at 242 nm. The RrF_1-ATPase was inactivated by stepwise additions of 50 μM DEPC added at 10 min intervals as indicated by the arrows in the inset. At these intervals the absorption changes at 242 nm were recorded and aliquots were removed, diluted and assayed as in fig. 1.

each step DEPC was applied at a 100-fold lower concentration than the one used in fig. 2, it did not interact with tyrosine and thus caused a specific modification of histidine residues in the RrF_1-ATPase. When the percent residual Ca^{2+}-ATPase activity of RrF_1 was plotted against the number of histidine residues modified, a linear line was obtained for up to 40% inactivation (fig. 5). When this initial portion of the curve is extrapolated to zero activity a stoichiometry of somewhat more than 2 histidines modified RrF_1 molecule is obtained. Further addition of more DEPC results, however, in modification of additional histidine residues that do not seem te be involved with the RrF_1 catalytic activity.

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