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Essential Histidyl Residues of Ferredoxin–NADP$^+$ Oxidoreductase Revealed by Diethyl Pyrocarbonate Inactivation†

Néstor Carrillo and Rubén H. Vallejos*

ABSTRACT: Diethyl pyrocarbonate inhibited diaphorase activity of ferredoxin–NADP$^+$ oxidoreductase with a second-order rate constant of 2 $mM^{-1}\cdot min^{-1}$ at pH 7.0 and 20 °C, showing a concomitant increase in absorbance at 242 nm due to formation of carbethoxyhistidyl derivatives. Activity could be restored by hydroxylamine, and the pH curve of inactivation indicated the involvement of a residue having a pK_a of 6.8. Derivatization of tyrosyl residues was also evident, although with no effect on the diaphorase activity. Both NADP$^+$ and NADPH protected the enzyme against inactivation, suggesting that the modification occurred at or near the nucleotide binding domain. The reductase lost all of its diaphorase activity after about two histidine residues had been blocked by the reagent. In differential-labeling experiments with NADP$^+$ as protective agent, it was shown that diaphorase inactivation resulted from blocking of only one histidyl residue per mole of enzyme. Modified reductase did not bind pyridine nucleotides. Modification of the flavoprotein in the presence of NADP$^+$, i.e., with full preservation of diaphorase activity, resulted in a significant impairment of cytochrome *c* reductase activity, with a second-order rate constant for inactivation of about 0.5 $mM^{-1}\cdot min^{-1}$. Reversal by hydroxylamine and spectroscopic data indicated that this second residue was also a histidine. Ferredoxin afforded only slight protection against this inhibition. Conversely, carbethoxylation of the enzyme did not affect complex formation with the ferrosulfoprotein. Redox titration of the modified reductase with NADPH and with reduced ferredoxin suggested that the second histidine might be located in the electron pathway between FAD and ferredoxin. On the basis of these results, two different types of essential histidyl residues can be distinguished in ferredoxin–NADP$^+$ oxidoreductase: One of them appears to be related with the nucleotide binding site, presumably behaving as a positive counterpart for the anionic molecule of NADP$^+$. The second, less reactive, histidine residue may be involved in the electron transport between ferredoxin and the flavin moiety.

Ferredoxin–NADP$^+$ oxidoreductase (EC 1.18.1.2) is a FAD[1]-containing enzyme that operates as the terminal acceptor of the photosynthetic electron transport chain in algae and higher plants. The membrane-bound flavoprotein catalyzes the reversible reduction of NADP$^+$ by ferredoxin (Shin & Arnon, 1965). Quite recently, Shahak et al. (1981) have suggested that it might also participate in the cyclic electron flow around photosystem I. The enzyme was initially described as a NADPH-specific diaphorase by Avron & Jagendorf (1956) and first isolated in a crystalline form by Shin et al. (1963).

The flavoprotein was shown to be located on the outer surface of the thylakoid membrane is a wide variety of photosynthetic organisms (Berzborn, 1968; Bohme, 1978; Rowell et al., 1981) with its catalytic site facing the chloroplast stroma. The attachment to, or solubilization from, the membrane results from a compromise between van der Waals attractive forces and Coulombic repulsion (Carrillo & Vallejos, 1982). The membrane-bound form of the enzyme undergoes a light-driven conformational change (Carrillo et al., 1980; Wagner et al., 1982) that may play a role in the activation of the photosynthetic electron transport during dark–light transitions (Satoh & Katoh, 1980; Carrillo et al., 1981b; Satoh, 1981; Carrillo & Vallejos, 1983b).

In addition to its physiological role in electron transport, the reductase, either in its soluble or membrane-bound form, is able to mediate several other reactions, including the oxidation of NADPH by artificial electron acceptors (diaphorase

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; Fd, ferredoxin; DCPIP, 2,6-dichlorophenolindophenol; FeCy, potassium ferricyanide; FAD, flavin adenine dinucleotide; NADP$^+$, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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activity) or by the ferredoxin–cytochrome *c* system (cytochrome *c* reductase activity). Rather little is known about the molecular mechanism of these two reactions, although the participation of a semireduced FAD intermediate has been established for the diaphorase (Massey et al., 1970). The investigation of the amino acid residues involved directly in the catalytic process is therefore of special interest.

Chemical-modification experiments carried out with the spinach enzyme revealed the existence of at least three different types of functional cysteinyl residues. One of them participates in a pH-dependent activation of the diaphorase activity (Davis & San Pietro, 1977). Modification of other SH groups results in release of tightly bound FAD (Zanetti & Forti, 1969) and impairment of ferredoxin binding (Valle et al., 1982).

There is also additional evidence concerning the presence in the enzyme of essential lysyl (Zanetti, 1976) and arginyl residues (Bookjans & Böger, 1978; Zanetti et al., 1979), as well as a carboxyl group (Carrillo et al., 1981a). The three amino acid side chains appear to behave as molecular counterparts for $NADP^+$ at the nucleotide binding site of the reductase.

The purpose of the present study is to get further information on the amino acids involved in the catalytic process of ferredoxin–$NADP^+$ reductase. Since a number of flavin-dependent enzymes have also been shown to contain histidyl residues at the active site (Steemkamp et al., 1974; Cousineau & Meighen, 1976; Cromartie, 1981; Phelps & Hatefi, 1981), the effect of chemical modification of ferredoxin–$NADP^+$ reductase with diethyl pyrocarbonate, a relatively specific histidine reagent (Melchior & Fahrney, 1970), has been investigated. These studies have provided evidence concerning the existence of two different essential histidyl residues: one of them being involved in binding of $NADP^+$ and the second one in the electron pathway between ferredoxin and FAD.

Experimental Procedures

Materials. Diethyl pyrocarbonate (ethoxyformic anhydride) was obtained from Sigma and stored desiccated near 0 °C to minimize hydrolysis. All stock solutions were prepared in absolute ethanol, and the molar concentration was determined by reaction of diethyl pyrocarbonate ($\simeq 10^{-4}$ M) with 1 mM histidine in 50 mM phosphate buffer, pH 7.0. The increase in absorbance at 242 nm was measured and the concentration calculated by using an extinction coefficient ϵ_{242} = 3.2 $mM^{-1}\cdot cm^{-1}$ (Miles, 1977). Spinach ferredoxin was purified according to the procedure of Buchanan & Arnon (1971).

Hydrogenase was prepared from *Desulfovibrio desulfuricans* cells, essentially as reported by Shin & Arnon (1965). Initial inocula were kindly povided by Dr. J. G. Zeikus, Department of Bacteriology, University of Wisconsin, Madison, WI, and by Dr. Meyer Wolin, Research Laboratories, Department of Health of New York State, Albany, NY.

The acetone powder resulting from the cells grown in 10 L of medium (20 g) was extracted twice with 100 mL of 50 mM Tris buffer, pH 8.0. The extract was applied to a DEAE-cellulose column equilibrated with 150 mM Tris buffer, pH 7.3. The red band containing active hydrogenase was eluted from the column with 200 mM Tris buffer, pH 7.3, and was used as the hydrogenase preparation. The presence of cytochrome c_3 did not interfere with the reactions that were investigated (Shin & Arnon, 1965).

The hydrogenase activity of each enzyme preparation was assayed as described by Shin & Arnon (1965), by measuring the reduction of benzylviologen at pH 8.0 under 1 atm of H_2. One unit was defined as the amount of enzyme that gave a change in absorbance of 1.0 per minute at 600 nm. Stock solutions contained about 200 units/mL and were stored frozen under liquid nitrogen.

NADP(H), glucose 6-phosphate, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, and cytochrome *c* were from Sigma. All other reagents were of analytical grade.

Enzyme Purification and Assay. Ferredoxin–$NADP^+$ oxidoreductase was purified from spinach chloroplasts as recently described (Carrillo & Vallejos, 1983a) by employing affinity chromatography on Cibacron Blue-Sepharose. The enzyme was removed from the dye-substituted Sepharose column by 10 volumes of eluent in a linear gradient: either $NADP^+$ (0–10 mM) or KCl (0.1–0.5 M) was employed with similar effectiveness in recovery and purification. The purified reductase had a specific activity between 110 and 150 μmol of FeCy·$mg^{-1}\cdot min^{-1}$ for the diaphorase assay and showed a single protein band in polyacrylamide gel electrophoresis, indicating that the enzyme was greater than 95% homogeneous by this criterion.

The diaphorase assay, with potassium ferricyanide as electron acceptor, was performed as previously described (Carrillo et al., 1980), while the ferredoxin-dependent cytochrome *c* reductase activity was determined by the method of Shin (1971).

Protein Determination. Both ferredoxin and ferredoxin–$NADP^+$ reductase concentrations were determined spectrophotometrically with extinction coefficients of ϵ_{420} = 9.7 $mM^{-1}\cdot cm^{-1}$ for ferredoxin (Buchanan & Arnon, 1971) and ϵ_{456} = 10.7 $mM^{-1}\cdot cm^{-1}$ for the flavoprotein (Forti et al., 1970). In some special cases, particularly during the purification of the bacterial hydrogenase, total protein concentration was determined by the method of Lowry et al. (1951).

Reaction with Diethyl Pyrocarbonate. Reductase (0.5–1 μM) was incubated at 20 °C in 50 mM phosphate buffer, pH 7.0, with different concentrations of diethyl pyrocarbonate, as indicated in the legends to tables and figures. The reaction was stopped at the desired extent of inactivation by the addition of 10%, by volume, of 0.1 M histidine, and the modified enzyme was assayed for activity in aliquots of 20 μL.

The number of histidine residues that reacted with diethyl pyrocarbonate was calculated by the change in absorbance at 242 nm with ϵ_{242} = 3.2 $mM^{-1}\cdot cm^{-1}$ (Miles, 1977). Spectra were recorded with an Aminco DW 2A spectrophotometer equipped with a Midan T microprocessor.

Complex Formation of Ferredoxin–$NADP^+$ Reductase. The fluorometric measurements of the complex formation between ferredoxin–$NADP^+$ reductase and $NADP^+$ were carried out essentially as described by Shin (1973) on a Perkin-Elmer 650-40 spectrofluorometer. Prior to the measurements, the flavoprotein preparations were filtered through Sephadex G-25 in order to remove the excess of reagents and free FAD. Complex formation with ferredoxin was measured by difference spectroscopy, following the procedure of Foust et al. (1969).

Oxidation–Reduction Titrations. A Thunberg-type cuvette with two side arms was used for all anaerobic experiments. Reduction of FAD by ferredoxin was performed essentially as described by Shin & Arnon (1965). The cuvette compartment contained the reaction mixture, including the reductase ($\simeq 5$ μM), 4 mM cysteine, and 0.8 mM $FeSO_4$ in 50 mM Tris buffer, pH 8.0. One of the side arms contained ferredoxin and 40 units of hydrogenase, whereas the other one contained the $NADP^+$. The entire system was flushed for about 30 min with hydrogen gas that was purified with KOH to remove possible carbon dioxide contamination. Before the contents of the first side arm were tipped into the cuvette

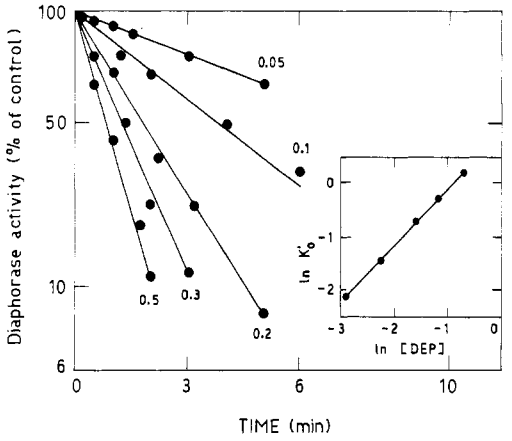

FIGURE 1: Kinetics of inactivation of diaphorase activity of ferredoxin–NADP$^+$ oxidoreductase by diethyl pyrocarbonate. Reductase (0.8 μM) was incubated with different concentrations of the reagent, and diaphorase activity was assayed in aliquots as described in the test. The numbers on the slopes indicate diethyl pyrocarbonate concentration in millimolar. Control activity was 138 μmol of FeCy·mg^{-1}·min^{-1}. (Inset) Apparent order with respect to reagent concentration for the reaction between the reductase and diethyl pyrocarbonate. The observed pseudo-first-order rate constant (K_0') values were calculated from the data of Figure 1 and plotted as shown.

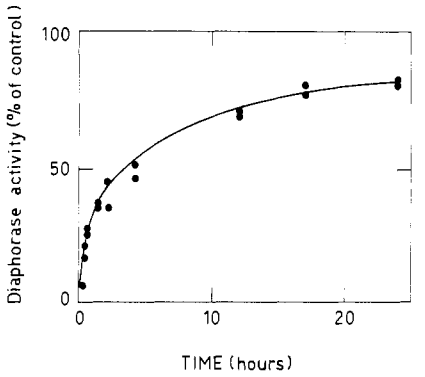

FIGURE 2: Reversal of diethyl pyrocarbonate inactivation of ferredoxin–NADP$^+$ reductase by hydroxylamine. Reductase (4 μM) was incubated with 0.2 mM diethyl pyrocarbonate for 30 min as described in the text. Then, the mixture was diluted with an equal volume of 1 M hydroxylamine, adjusted to pH 7.0 with KOH, and incubated at approximately 0 °C. Aliquots were removed and assayed for enzymatic activity at the times shown. No inhibition of the native reductase by hydroxylamine was observed in these conditions. Control activity was 122 μmol of FeCy·mg^{-1}·min^{-1}.

compartment, the absorbance at 456 nm was recorded. Final concentrations of ferredoxin and NADP$^+$ in the reaction mixture (2.6 mL) were 10 μM and 0.4 mM, respectively.

For the reduction of the flavoprotein with NADPH, the cuvette compartment contained approximately 5 μM ferredoxin–NADP$^+$ reductase in 50 mM Tris buffer, pH 8.0. The first side arm contained NADP$^+$ (7 μM), glucose 6-phosphate (0.5 mM), and glucose-6-phosphate dehydrogenase (2 units/mL) in the same buffer. The second side arm contained the electron acceptor DCPIP. The cuvette was flushed with nitrogen gas for about 30 min prior to any spectral measurement and closed with a rubber cap. After the initial absorbance was recorded, the contents of the first side arm were tipped into the cuvette, and the reaction was allowed to proceed until no further change in absorbance could be detected. Before the solution of the second side arm was tipped, 10 units of phosphoglucose isomerase was added from a syringe through the rubber cap of the cuvette. The mixture was incubated for 10 min under stirring, with no changes in its spectral properties. Final concentration of DCPIP was 50 μM.

When a modified reductase was assayed for its redox properties, the enzyme was previously filtered through Sephadex G-25, according to Penefsky (1977) in order to eliminate the excess of reagent and protective agents. All determinations were made at 20 °C in an Aminco DW 2A spectrophotometer.

Results

Inactivation of Diaphorase Activity by Diethyl Pyrocarbonate. Ferredoxin–NADP$^+$ reductase lost essentially all of its diaphorase activity when treated with diethyl pyrocarbonate. Semilogarithmic plots of residual activity vs. time (Figure 1) were linear, indicating that the inactivation was first order with respect to enzyme concentration.

The rate of inactivation was a function of the diethyl pyrocarbonate concentration, and a double-reciprocal plot of the apparent first-order rate constants, determined from the slopes of the lines of Figure 1, vs. the corresponding concentration of reagent gave a straight line that failed to yield a finite intercept, this result suggesting a simple bimolecular reaction. However, the possible existence of an intermediate protein–inhibitor complex with a very high dissociation constant cannot be excluded. From these data a second-order rate constant of 2 mM^{-1}·min^{-1} may be calculated. A double-logarithmic plot of the half-times of inactivation against reagent concentration (Levy et al., 1963) yields a reaction order of 1 with respect to inhibitor (inset of Figure 1).

Although the reaction of diethyl pyrocarbonate with proteins is relatively specific at pH 7.0 for histidyl residues (Miles, 1977), other nucleophilic groups may also be modified in weakly alkaline media (Melchior & Fahrney, 1970; Wells, 1973; Burstein et al., 1974). Consequently, the identity of the amino acid residue(s) in the reductase, whose modification results in the loss of diaphorase activity, was investigated by (a) treating of the inactivated enzyme with hydroxylamine, (b) studying the effect of pH on the rate of inactivation, and (c) following changes in the absorbance at 242 and 280 nm of the flavoprotein upon modification.

Treatment of carbethoxy reductase with NH_2OH results in restoration of about 80% of the original activity after a prolonged incubation (Figure 2). Since NH_2OH is known to remove the carbethoxy group from modified histidyl and tyrosyl residues, but does not cleave the more stable carbethoxylysyl and carbethoxysulfhydryl bonds (Miles, 1977), the inactivation of reductase by diethyl pyrocarbonate can be tentatively attributed to the modification of histidyl and/or tyrosyl residues.

Further evidence for the modification of histidine(s) comes from the study of the rate of inactivation as a function of pH. The pseudo-first-order rate constants for inactivation were determined at different pH and plotted against [H$^+$], according to the equation of Cousineau & Meighen (1976):

$$1/K_0' = 1/(k_2[\mathrm{I}]) + [\mathrm{H}^+]/(k_2K_a[\mathrm{I}])$$

where K_a is the apparent acidic dissociation constant and k_2 is the second-order rate constant of formation of the covalent bond for the totally unprotonated form of the enzyme. The resulting curve is illustrated in Figure 3.

From the intercept and slope, the apparent pK_a of the inhibition was found to be 6.8, a value that is consistent with the modification of a histidyl residue. It should be pointed out that the straight line was only drawn through the points at acidic or neutral pH, since a curvature downward can clearly be observed above pH 7.5. The deviation from the linear behavior at alkaline pH reflects presumably modification of other essential residues and/or a more reactive conformation

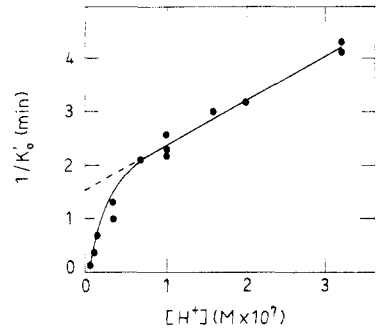

FIGURE 3: pH dependence of inactivation of ferredoxin–NADP$^+$ reductase by diethyl pyrocarbonate. All reactions were conducted at 20 °C in 50 mM phosphate buffer adjusted to the desired pH with NaOH. The pseudo-first-order rate constant (K_0') values for each pH were obtained from the half-times of inactivation of the diaphorase activity with 0.2 mM diethyl pyrocarbonate and plotted according to Cousineau & Meighen (1976).

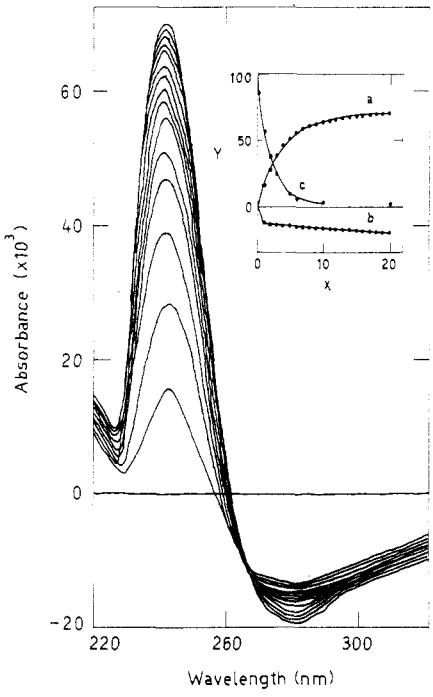

FIGURE 4: Ultraviolet difference spectra for inactivation of ferredoxin–NADP$^+$ oxidoreductase by diethyl pyrocarbonate. A 2.2-mL sample of reductase (10 μM) was divided equally between the sample and reference cuvettes in the spectrophotometer, and the base line was recorded. Diethyl pyrocarbonate (0.25 mM) was added to the sample cuvette and an equivalent volume of ethanol to the reference cuvette. Difference spectra were recorded at every 1 min after the addition of reagent. The absorbance changes at 242 nm increased with time. (Inset) Time course of the absorbance changes at 242 (curve a) and 280 nm (curve b) and the loss of diaphorase activity (curve c) upon modification of ferredoxin–NADP$^+$ reductase with diethyl pyrocarbonate. The x axis indicates the time of incubation in minutes, whereas the y axis represents either the magnitude of the absorbance changes ($\times 10^3$) or the percentage of residual activity. The initial diaphorase activity was 135 μmol of FeCy·mg^{-1}·min^{-1}.

of the reductase in response to a pH increase, as previously reported (Davis & San Pietro, 1977).

Carbethoxylation of histidyl residues can be distinguished from that of tyrosyl ones by difference spectroscopy. The reaction will result in an increase in absorbance at 242 nm if histidines are modified (Ovádi et al., 1967) and a decrease in absorbance at 280 nm if *O*-carbethoxytyrosyl bonds are formed (Muhlrad et al., 1969). The dependence of the difference absorbance of reductase at 242 and 280 nm upon inactivation with diethyl pyrocarbonate (Figure 4) indicates that both types of residues are modified. However, while a rapid modification

Table I: Protection by Nucleotides of Ferredoxin-NADP$^+$ Oxidoreductase against Diethyl Pyrocarbonate Inactivation[a]

additions during modification	diaphorase activity (μmol of FeCy·mg^{-1}·min^{-1})
none	13 (11)
NADP$^+$	118 (97)
NADPH	102 (84)
NAD$^+$	89 (73)
NADH	80 (66)
2′-AMP	85 (70)
ADP	50 (41)
ATP	35 (29)
UDP	18 (15)
IMP	14 (11)
IDP	12 (10)
dADP	40 (33)

[a] Reductase (0.5 μM) was modified with 0.2 mM diethyl pyrocarbonate for 5 min in 50 mM phosphate buffer, pH 7.0, with the additions stated. Nucleotides, when present, were 2 mM. Diaphorase activity was determined as described in the text. Numbers in parentheses indicate percentage of inactivation. Activity of the control was 122 μmol·mg^{-1}·min^{-1}.

of tyrosine residue(s) was observed within the first minute of reaction, followed by a much slower incorporation (inset of Figure 4), the increase in absorbance at 242 nm showed a rather different kinetics and a good correlation with the loss of enzymatic activity (see below). On the other hand, treatment of the reductase with the tyrosyl reagent NBD-Cl resulted in significant incorporation of the modifier to the flavoprotein (Ferguson et al., 1975) with no loss of diaphorase activity. This treated flavoprotein could be further inactivated by diethyl pyrocarbonate, and in this case, no absorbance decrease in the region of 280 nm could be detected (data not shown).

Protection Experiments. The effects of several nucleotides on the inhibition of diaphorase activity by diethyl pyrocarbonate are shown in Table I. Either NADP$^+$ or NADPH afforded complete protection to the reductase against inactivation. NAD(H) and 2′-AMP protected only partially while other nucleotides, when tested at the same concentration as NADP$^+$ and NADPH, afforded little or no protection at all.

Determination of the K_d for NADP$^+$ as protective agent by the procedure of Scrutton & Utter (1965) gave a value of 50 μM, similar to that published by Zanetti (1976) for dansyl chloride inactivation. The straight line obtained in this plot passed through the origin, which implies that the flavoprotein–NADP$^+$ complex cannot be inactivated by diethyl pyrocarbonate.

Miles (1977) reported that diethyl pyrocarbonate can give a ring-opening reaction with the imidazole ring of adenosine when fairly high concentrations of reagent are employed. In order to rule out the possibility that the protection patterns observed were due to reaction of diethyl pyrocarbonate with NADP$^+$, a solution of nucleotide at 2 mM concentration was treated with 0.5 mM reagent for 30 min. No change in the absorbance at 280 nm could be detected during this period, which should have indicated cleavage of the adenine moiety. Furthermore, when compared with native NADP$^+$ in its ability to oxidize glucose 6-phosphate via glucose-6-phosphate dehydrogenase, this treated nucleotide gave an identical increase in the absorbance at 340 nm, indicative of NADPH formation. Finally, either native or treated NADP$^+$ was equally effective in protecting the enzyme against inactivation by diethyl pyrocarbonate. In view of these evidences, we consider that the protection experiments described above must be taken as valid.

The number of histidyl residues that had reacted either in

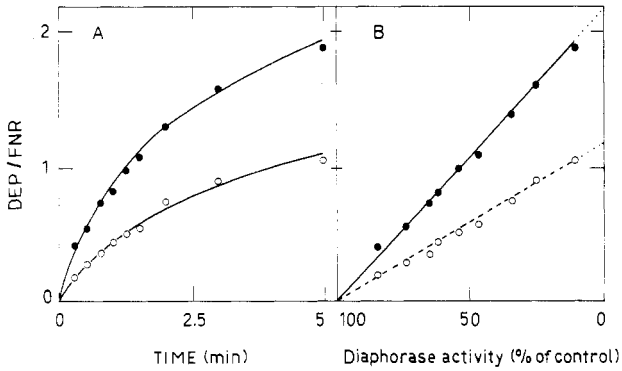

FIGURE 5: Relationship of diaphorase activity to number of histidine residues modified by diethyl pyrocarbonate. Difference spectra for the inactivation in the absence of NADP$^+$ (●) were recorded essentially as in Figure 4. Reductase (6.8 μM) was incubated with 0.23 mM diethyl pyrocarbonate. At the corresponding intervals, the *N*-carbethoxyhistidines formed and the diaphorase activity were determined as described in the test. Modification in the presence of 1.5 mM NADP$^+$ (○) was carried out under the same conditions, but the reductase concentration was adjusted to 25 μM in order to obtain a better signal. Excess of NADP$^+$ was removed by gel filtration through Sephadex G-25 according to Penefsky (1977). The absorbance changes at 242 nm were recorded against a reference of native enzyme at the same concentration. The number of *N*-carbethoxy groups formed per mole of enzyme was plotted as a function of time (A) and of residual activity (B). In the last case, the experimental points obtained in the presence of NADP$^+$ (dashed lines, open circles) showed no inactivation of the diaphorase activity; therefore, they were plotted at the inactivation achieved at the same incubation time in the absence of nucleotide.

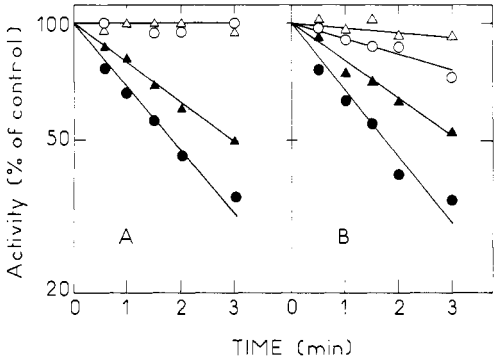

FIGURE 6: Effect of diethyl pyrocarbonate on diaphorase (A) and cytochrome *c* reductase (B) activities of ferredoxin–NADP$^+$ oxidoreductase. The enzyme (0.5 μM) was incubated with 0.23 mM diethyl pyrocarbonate in the absence (●) or in the presence of 2 mM NADP$^+$ (○), 30 μM ferredoxin (▲), or a combination of both (△). Other experimental details are described under Experimental Procedures. Activities of the control were 131 and 84 μmol·mg^{-1}·min^{-1} for diaphorase and cytochrome *c* reductase, respectively.

the absence or in the presence of NADP$^+$ was calculated from the change in absorbance at 242 nm, by using ϵ_{242} = 3.2 mM^{-1}·cm^{-1} for the *N*-carbethoxyhistidine (Miles, 1977) and a molecular mass of 36 kdalton for the flavoprotein (Sheriff et al., 1980). Figure 5A shows the time course of diethyl pyrocarbonate incorporation in the presence and in the absence of NADP$^+$. The same experimental points are plotted in Figure 5B as the number of modified residues vs. the residual activity of the unprotected enzyme. Extrapolation of the least-squares lines to zero enzymatic activity indicates that two histidyl residues per FAD were modified before complete inactivation was achieved, when the reaction was carried out in the absence of nucleotide. However, NADP$^+$ prevented incorporation of one carbethoxy group per FAD, with total preservation of diaphorase activity (Figure 5B).

The interpretation of the data was confirmed by using the approach of Tsou (1962), which indicates that only one of the two modified histidines is actually "essential" for the enzymatic activities of ferredoxin–NADP$^+$ reductase involving oxidation of NADPH or reduction of NADP$^+$ (not shown).

Effect of Diethyl Pyrocarbonate on Cytochrome c Reductase Activity. In the preceding paragraphs, we have demonstrated the existence of a histidyl group essential for the diaphorase activity of ferredoxin–NADP$^+$ reductase. However, this reaction is believed to involve only the electron pathway between (NADP)H and FAD. In an attempt to elucidate the functionality of the other residues modified, we studied the effect of diethyl pyrocarbonate on the cytochrome *c* reductase activity, a ferredoxin-mediated process that involves the electron transfer from the ferrosulfoprotein to NADPH.

The effects of diethyl pyrocarbonate on both diaphorase and cytochrome *c* reductase are summarized in Figure 6. Inactivation of the two reactions in the absence of substrates followed essentially the same kinetics with a K_0' of about 0.4 min^{-1} for a reagent concentration of 0.2 mM. Ferredoxin afforded partial protection, decreasing the K_0' to approximately 0.23 min^{-1} in both cases. Modification in the presence of NADP$^+$ resulted in a slow inhibition of the cytochrome *c* reduction rate (Figure 6B) without impairing diaphorase activity, whereas the presence of ferredoxin even in addition to NADP$^+$ during the modification did not result in complete protection of the cytochrome *c* reductase activity (Figure 6B). Full inactivation of this reaction in the presence of NADP$^+$ could be achieved with two successive additions of 0.25 mM reagent within 50 min of incubation (not shown).

These results suggest that a second residue different from the histidine of the nucleotide binding site may be involved in some other part of the catalytic process. The nature of this less reactive residue was investigated as before. Addition of hydroxylamine to partially inactivated enzyme led to complete recovery of cytochrome *c* reductase activity. Spectral changes accompanying inactivation indicated simultaneous modification of histidine and tyrosine residues. The possible participation of essential tyrosines in a ferredoxin-mediated reaction would agree with results obtained by Hasumi & Nakamura (1978), who reported on the existence of one or more tyrosyl residues at the ferredoxin binding site of the flavoprotein. They also showed that in the presence of ferredoxin, such residues become incapable of interacting with the solvent. At variance with these results, we found only a slight protection by ferredoxin against inactivation of the cytochrome *c* reductase (Figure 6). Specificity for histidines was also supported by the following evidence: (a) chemical modification of the reductase with the tyrosyl reagent NBD-Cl did not affect cytochrome *c* reductase activity; (b) this treatment totally prevented further modification of tyrosyl residues by diethyl pyrocarbonate; (c) the cytochrome *c* reductase activity of either native or NBD-modified reductase was similarly inactivated when treated with diethyl pyrocarbonate in the presence of NADP$^+$.

In view of these results, we conclude that the second essential group is more likely a histidine than a tyrosine. The poor protective effect showed by ferredoxin suggests that the residue might not be related to the binding site of ferredoxin. In order to check these assumptions, we have investigated the effect of the carbethoxylation of the enzyme on its ability to interact with ferredoxin and with NADP$^+$.

Complex Formation of Carbethoxylated Ferredoxin–NADP$^+$ Reductase with NADP$^+$ and with Ferredoxin. It has been reported (Shin, 1973) that ferredoxin–NADP$^+$ reductase undergoes an increase in the FAD fluorescence upon binding of NADP$^+$. This effect is likely due to a loosening of the

Table II: Oxidoreduction Titration of Native or Modified Ferredoxin-NADP$^+$ Oxidoreductase[a]

additions	ΔA_{456} ($\times 10^3$) control	DEP treated	NADP$^+$ protected	Fd protected
expt 1				
none	48	48	49	49
reduced Fd	22 (46)	35 (73)	33 (67)	33 (67)
NADP$^+$	47 (98)	36 (75)	48 (98)	34 (69)
expt 2				
none	45	46	46	44
NADPH	26 (58)	44 (96)	27 (59)	38 (86)
DCPIP	43 (96)	46 (100)	45 (98)	45 (102)

	residual activities after modification (μmol·min^{-1}·mg^{-1}) control	DEP treated	NADP$^+$ protected	Fd protected
diaphorase	111	17	114	32
cyt *c* reductase	87	13	46	24

[a] The flavoprotein (10 μM) was incubated at 20 °C with 0.23 mM diethyl pyrocarbonate for 5 min (DEP treated) in a medium containing 50 mM phosphate buffer, pH 7.0. Ferredoxin (Fd protected) and NADP$^+$ (NADP$^+$ protected), when present during modification, were 36 μM and 2 mM, respectively. The samples were filtered through Sephadex G-25 prior to any spectral measurement. Redox titrations were carried out in 50 mM Tris buffer, pH 8.0, in Thunberg-type cuvettes. Reductase concentrations in the main compartment of the cuvette were adjusted with buffer to give the initial values of absorbance indicated in the table before any addition (none). The anaerobic reduction of FAD by ferredoxin and reoxidation by NADP$^+$ (experiment 1) or by NADPH and reoxidation by DCPIP (experiment 2) were carried out as described in the text. Numbers in parentheses are the percentages of the initial values of absorbance.

prosthetic group from the apoprotein. We have used this experimental approach to evaluate the complex-forming properties of the enzyme.

The titration curves of native or modified ferredoxin–NADP$^+$ reductase with NADP$^+$ are shown in Figure 7. The amplitude of the fluorescence increase in the native enzyme is related to the NADP$^+$/reductase ratio via a nearly perfect hyperbolic behavior with an apparent stoichiometry of 1.14 and a K_d of 7–15 μM, whereas the carbethoxylated enzyme showed a drastic inhibition in its ability to interact with the nucleotide. The K_d for NADP$^+$ reported here is considerably lower than that obtained from protection experiments by the Scrutton–Utter approach (i.e., 50 μM). The discrepancy may be due to the higher ionic strength of the medium in the last case (50 mM phosphate against 10 mM in the fluorometric titration). Such an effect of the ionic strength on the affinity of the reductase for its substrates has been already reported by several authors (Foust et al., 1969; Keirns & Wang, 1972; Zanetti, 1976). Complex formation between the reductase and ferredoxin, as measured by difference spectroscopy (Foust et al., 1969), was hardly affected by the modification of the flavoprotein with diethyl pyrocarbonate, no matter if it had been carried out in the absence or in the presence of saturating amounts of NADP$^+$ (data not shown).

According to these data, the inhibitory effect of diethyl pyrocarbonate on cytochrome *c* reductase activity documented in Figure 6 may be attributed to an effect of the reagent on the electron transfer through the reductase polypeptide backbone and not to alterations in the binding of ferredoxin. The precise localization of this second histidyl residue requires the partition of the overall reaction into two hemireactions, that is, from ferredoxin to the FAD moiety and from the prosthetic group toward NADP$^+$, since the inhibition of cytochrome *c* reduction might be the result of blockade of either of these two electron pathways. In order to distinguish between these possibilities, redox titrations of FAD in native and modified ferredoxin–NADP$^+$ reductase were carried out under anaerobic conditions, employing different electron acceptors and donors.

Redox Titration of Ferredoxin–NADP$^+$ Reductase. The flavoprotein can be fully reduced anaerobically by either ferredoxin or NADPH, in both cases in the presence of a regenerating system (Shin & Arnon, 1965; Keirns & Wang, 1972). Complete reduction results in disappearance of the flavin peak at 456 nm, with a decrease in the extinction coefficient from 10.7 to 6.1 mM^{-1}·cm^{-1} (Forti et al., 1970).

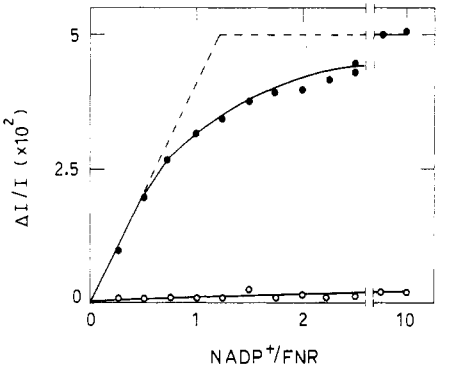

FIGURE 7: Fluorometric determination of complex between NADP$^+$ and native (●) or modified (○) ferredoxin–NADP$^+$ oxidoreductase (10 μM) in 10 mM phosphate buffer, pH 7.0. The enzyme was modified by 0.23 mM diethyl pyrocarbonate for 30 min (residual activity about 6% of control) and filtered through Sephadex G-25 prior to any fluorescence determination. The excitation wavelength was set at 450 nm and the emission wavelength at 526 nm. All data were corrected for dilution. The broken line indicates a theoretical curve, corresponding to an apparent stoichiometry of 1.14 and a dissociation constant (K_d) of 7 μM.

Reduction of purified ferredoxin–NADP$^+$ reductase by ferredoxin can proceed in the presence of hydrogen gas and a bacterial hydrogenase as a source of reducing power. In the absence of electron acceptors, the fully reduced form of FAD is generated. On the other hand, Shin & Arnon (1965) demonstrated that the enzyme, when first reduced by reduced ferredoxin, can be subsequently reoxidized by the addition of excess NADP$^+$. By means of this procedure, the reaction can be conveniently divided into two steps.

We have analyzed the redox changes of native ferredoxin–NADP$^+$ reductase and of reductase modified by diethyl pyrocarbonate under three different modes: in the absence of substrates, in the presence of 2 mM NADP$^+$, and in the presence of 36 μM ferredoxin. In the following, we name these modified samples as "unprotected", "NADP$^+$ protected", and "ferredoxin protected", respectively. The results are summarized in Table II, together with the data of residual activity for both the diaphorase and cytochrome *c* reductase activity.

Table II (experiment 1) shows that the electron transfer from reduced ferredoxin to FAD was partially impaired by treatment of the enzyme with diethyl pyrocarbonate. The results correlate rather well with the inhibition of cytochrome *c* reductase in the presence of NADP$^+$. The extent of this inhibition was only slightly altered by the addition of saturating amounts of ferredoxin or NADP$^+$ during the modification.

Conversely, reoxidation of the reduced flavoprotein by NADP$^+$ could only be attained with the NADP$^+$-protected enzyme, while not with the unprotected or the ferredoxin-protected one. These results are consistent with the localization of the second essential histidine in the pathway between ferredoxin and FAD.

It has been already mentioned that the electron transport from ferredoxin to NADP$^+$ is reversible. Therefore, anaerobic reduction of the flavoprotein by NADPH was carried out in the presence of a NADPH-regenerating system (glucose 6-phosphate and glucose-6-phosphate dehydrogenase). Reoxidation experiments employing the diaphorase acceptor DCPIP were attempted. For this purpose, an excess of phosphoglucose isomerase was added to the reaction mixture prior to the oxidant, in order to convert glucose 6-phosphate into fructose 6-phosphate. This reaction prevents regeneration of NADPH by the glucose 6-phosphate dehydrogenase system and allows reoxidation to occur. The controls in experiment 2 of Table II show that FAD reoxidation was complete under these conditions. Reduction of the flavin group of ferredoxin–NADP$^+$ reductase by NADPH was affected by diethyl pyrocarbonate in a manner similar to that of the diaphorase activity (Table II, experiment 2). Interestingly, reoxidation by the diaphorase acceptor DCPIP was complete in the native and the three modified flavoprotein samples, suggesting that this electron acceptor may be reduced by a different redox mechanism, without involving the essential histidine located in the electron pathway toward ferredoxin.

Discussion

The reaction of diethyl pyrocarbonate with proteins can lead to the modification of several amino acid side chains, such as imidazole, phenolates, sulfhydryls, α- and ϵ-amines, and guanidino groups (Muhlrad et al., 1969; Melchior & Fahrney, 1970). Modification of histidyl residues is believed to be rather specific below pH 7.0, although carbethoxylation of tyrosyl and amino groups has also been reported (Melchior & Fahrney, 1970; Wells, 1973; Burstein et al., 1974). However, inactivation of proteins by modification of imidazole groups with diethyl pyrocarbonate has generally resulted in second-order rate constants greater than 0.1 $mM^{-1}\cdot min^{-1}$, compared with the rates of modification of other residues, which are at least 10 times lower (Holbrook & Ingram, 1973; Wells, 1973). If the rate constant for inactivation at pH 7.0 is sufficiently high, it may be reasonably concluded that modification of hystidyl residues is responsible.

In the present experiments, ferredoxin–NADP$^+$ reductase was inactivated in 50 mM phosphate buffer, pH 7.0, at 20 °C with diethyl pyrocarbonate (Figure 1). Although carbethoxylation of a phenolate of tyrosine was also evident from the inspection of the spectroscopic data of Figure 4, we think that inhibition of the diaphorase activity was due to the modification of histidyl residues, according to the following evidence: (a) the correlation between the loss of enzymatic activity and the increase in the absorbance at 242 nm, indicative of the formation of *N*-carbethoxyhistidine (Figure 5); (b) the reversal of the inactivation by hydroxylamine (Figure 2); (c) the dependence of the reaction rate on a residue with a pK_a equal to 6.8, in agreement with the p*K* values reported for histidyl residues in other proteins modified with diethyl pyrocarbonate (Holbrook & Ingram, 1973; Cousineau & Meighen, 1976) (Figure 3); (d) a high second-order rate constant for inactivation of 2 $mM^{-1}\cdot min^{-1}$, a rate as large as those observed for the hyperreactive histidyl residues in pig heart lactate dehydrogenase (Holbrook & Ingram, 1973) and yeast alcohol dehydrogenase (Dickenson & Dickinson, 1975) at pH 6.0. In addition, prior modification of the tyrosyl residues with NBD-Cl had no effect on the reaction of ferredoxin–NADP$^+$ reductase with diethyl pyrocarbonate.

Spectroscopic investigation of the inactivation process indicated that 2.05 histidine residues are modified before complete loss of activity is achieved (Figure 5). Evaluation of the relationship between the activity remaining in the enzyme and the number of residues modified by the reagent, performed by the procedure of Tsou (1962), showed that only one of the reactive histidines is actually essential for catalysis. The flavoprotein contains a total of five histidines per FAD, as revealed by amino acid analysis (Hasumi & Nakamura, 1978).

Both NADP$^+$ and NADPH fully protected the enzyme from inactivation by diethyl pyrocarbonate (Table I) and prevented the incorporation of a single carbethoxy group into the modified reductase (Figure 5). Moreover, the derivatized flavoprotein was not capable of interacting with NADP$^+$ (Figure 7). These results suggest that the essential histidyl residue blocked by diethyl pyrocarbonate is located at the nucleotide binding site of the enzyme.

If the reductase was treated with diethyl pyrocarbonate in the protected mode, i.e., when the nucleotide binding site was protected by the presence of NADP$^+$ during the incubation with the reagent, the modified flavoprotein had full diaphorase activity whereas its cytochrome *c* reductase activity was significantly impaired (Figure 6). This result suggests the existence of a second essential group in ferredoxin–NADP$^+$ reductase. The reversion by hydroxylamine and the effect of tyrosyl reagents suggest that this second residue is also a histidine, whose modification should account for about 20% of the total carbethoxyimidazole groups formed upon complete inactivation of diaphorase activity.

This additional, less reactive, residue appeared not to be related with binding of ferredoxin, in view of the poor protection afforded by the sulfoferroprotein against inactivation (Figure 6). Conversely, carbethoxylation of the flavoprotein did not affect its complex-forming properties with ferredoxin.

X-ray diffraction of the reductase–NADPH complex at 3.7-Å resolution (Sheriff & Herriot, 1981) revealed that the enzyme consists of two domains, one containing the nucleotide binding site at its surface (Wagner et al., 1981) and the other one the FAD moiety and presumably also the ferredoxin domain. This model implies that the electron transfer from ferredoxin to NADP$^+$ may include a pathway through the polypeptide backbone, thus involving the participation of one or more amino acid side chains in the redox mechanism. Following this hypothesis, we find that the results obtained in the oxidation–reduction experiments are consistent with the location of the less reactive histidine in the electron pathway between ferredoxin and FAD (Table II). Similar results have been obtained by Wagner et al. (1981) for the lysyl reagent eosin isothiocyanate. In addition, they also reported that the interaction of the reductase with NADP$^+$ causes conformational changes that are allosterically transmitted to the ferredoxin domain. Since the lower reactivity of the second histidine toward diethyl pyrocarbonate suggests a certain degree of inaccessibility of this residue, it might be speculated that the access of diethyl pyrocarbonate to this domain should

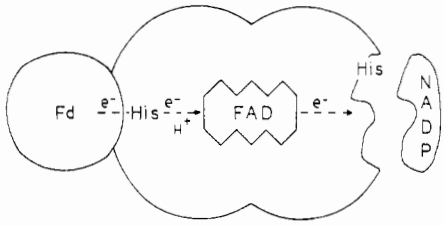

FIGURE 8: Schematic picture of distribution of essential histidine residues in ferredoxin–$NADP^+$ oxidoreductase.

be modulated by the interaction of $NADP^+$ with its binding site. However, the redox-titration experiments showed that the reduction of FAD by reduced ferredoxin (Table II) was inhibited by diethyl pyrocarbonate approximately to the same extent no matter whether the nucleotide was present during the modification or not. These results indicate that the accessibility of the second imidazole group was substantially unaffected by the conformational changes of the flavoprotein upon complex formation with $NADP^+$.

Modification of any or both of the two essential histidyl residues did not affect the reoxidation of the enzyme by the diaphorase acceptor DCPIP (Table II), suggesting that this oxidant may be reduced by a different mechanism. Kinetic data on the diaphorase reaction with either DCPIP or potassium ferricyanide as electron acceptor showed that this reaction follows a two-step (ping-pong) mechanism (Nakamura & Kimura, 1971). Moreover, Ricard et al. (1980) demonstrated that the reduced form of potassium ferricyanide binds nonspecifically to both the $NADP^+$ and the ferredoxin binding sites and perhaps to other places in the reductase molecule. According to these data, two hypotheses can be formulated: (a) The diaphorase acceptors are directly reduced by the FADH· moiety without involvement of the polypeptide backbone. (b) DCPIP is reduced at the $NADP^+$ binding site by the same mechanism that reduces the nucleotide. If this is so, then the essential histidine located in this domain would be involved only in $NADP^+$ binding and not in the reductive catalytic process.

Reduction of ferredoxin–$NADP^+$ reductase by ferredoxin has been measured in whole *Chlorella* cells by flash spectrophotometric techniques (Bouges-Bocquet, 1980). Under flash excitation of photosystem I, the half-time of formation of the neutral semiquinone FADH· is about 1 μs at 20 °C. This protonation of the flavin moiety is too rapid to be a diffusion process at physiological pH. It is necessary to conclude that the proton is already fixed close to the FAD molecule before the photochemical reaction. Histidyl residues are suitable candidates for this function in proton conduction, and their role as hydrogen ion carriers has been extensively discussed for several dehydrogenases (Nishino et al., 1980; Phelps & Hatefi, 1981; Vik & Hatefi, 1981). Therefore, we can speculate that the slow-reacting histidine may participate in the physiological proton transfer toward FAD during the photosynthetic electron flow through ferredoxin–$NADP^+$ reductase. Research is in progress to elucidate this point.

Conclusions

The interpretation of the results described in this paper is summarized in Figure 8. The drawing represents the ferredoxin–$NADP^+$ reductase, ferredoxin, $NADP^+$, and the essential histidines. The experimental data lead us to the following conclusions: (a) There are at least two types of essential histidyl residues in ferredoxin–$NADP^+$ oxidoreductase. (b) The fast-reacting residue is in close proximity of the binding site for $NADP^+$ and, therefore, also very close to the surface of the protein, presumably acting as a charge counterpart for the anionic molecule of $NADP^+$. (c) The slow-reacting histidine is located in the electron pathway between ferredoxin and FAD and appears to be partially buried in the polypeptide backbone. Its possible role in the proton conduction toward FAD remains to be confirmed.

Acknowledgments

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Registry No. NADP, 53-59-8; NADPH, 53-57-6; EC 1.18.1.2, 9029-33-8; L-histidine, 71-00-1; diethyl pyrocarbonate, 1609-47-8.

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β-Hydroxydecanoyl Thio Ester Dehydrase Does Not Catalyze a Rate-Limiting Step in *Escherichia coli* Unsaturated Fatty Acid Synthesis†

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ABSTRACT: The intracellular level of β-hydroxydecanoyl thio ester dehydrase, the product of the *fabA* gene of *Escherichia coli*, was increased by isolation of a putative promotor mutant (termed *fabAup*) or by molecular cloning of the wild-type *fabA* gene into plasmid pBR322. The *fabAup* and plasmid-carrying strains overproduced dehydrase by about 15- and 10-fold, respectively. The phospholipids of all strains that overproduced the dehydrase contained significantly higher levels of saturated fatty acids than isogenic strains producing a normal level of dehydrase. No increased levels of unsaturated fatty acids were observed. This result indicates that, although the dehydrase is required for unsaturated fatty acid synthesis, the level of dehydrase activity in wild-type cells does not limit the rate of unsaturated fatty acid synthesis. The introduction of a plasmid carrying the structural gene for β-ketoacyl acyl carrier protein synthase I into a *fabAup* strain overcame the effect of dehydrase overproduction on fatty acid composition.

Escherichia coli adjusts the fatty acid composition of its phospholipids in response to growth temperature [for review, see DeMendoza & Cronan (1983)]. This regulatory mechanism functions to lower the temperature of the order–disorder lipid phase transition and thus optimizes membrane function at lower temperatures (DeMendoza & Cronan, 1983). Thermal regulation is now known to be an intrinsic property of the fatty acid biosynthetic enzyme β-ketoacyl-ACP[1] synthase II (DeMendoza & Cronan, 1983). However, mutants lacking this enzyme, although completely defective in thermal regulation, synthesize a characteristic mix of saturated and unsaturated fatty acids. This result indicates the existence of an underlying mechanism regulating fatty acid composition that is independent of temperature. This mechanism is the subject of this paper.

The site of regulation of fatty acid composition in the absence of regulation by temperature has often been proposed to be exerted at the level of the acylation of *sn*-glycerol 3-phosphate to form phosphatidic acid, a key intermediate in phospholipid synthesis [for review, see Rock & Cronan (1982)]. However, acylation specificity is not absolute in vivo (Silbert, 1970), and thus, a major site of control must be at the level of fatty acid synthesis. The enzyme, β-hydroxydecanoyl thio ester dehydrase is responsible for the introduction of a cis double bond into the growing acyl chain to give the

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[1] Abbreviations: ACP, acyl carrier protein; NAC, *N*-acetylcysteamine; DNAC, 3-decynoyl-*N*-acetylcysteamine, Tet^R, tetracycline resistant.

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