The Specificity of Carboxyl Group Modification during the Inactivation of the *Escherichia coli* F$_1$-ATPase with Dicyclohexyl[14C]carbodiimide*

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When the *Escherichia coli* F$_1$-ATPase (EF$_i$) is inactivated with dicyclohexyl14Ccarbodiimide and then gel-filtered, about 1 g atom of 14C is bound per mol of enzyme. However, only 45% of this radioactivity remained bound to the protein when the subunits were isolated in the presence of 8 μm urea. The majority of the radioactivity which remained bound to the protein during subunit isolation was associated with the β subunit that contained 0.13 g atom of 14C per mol. A tryptic peptide derived from the labeled β subunit was isolated which contained the majority of the radioactivity bound to it. Based on the amino acid composition of this peptide, the amino acid compositions of radioactive fragments derived from it, and the amino acid sequence of the β subunit of EF$_i$, translated from the gene (Saraste, M., Gay, N. J., Eberle, A., Runswick, M. J., and Walker, J. E. (1981) Nucleic Acids Res. 9, 5287-5296 and Kanazawa, H., Kayano, T., Kyisu, T., and Futai, M. (1982) Biochem. Biophys. Res. Commun. 105, 1257-1264), it is concluded that the glutamic acid residue marked with an asterisk in the sequence Glu-Gly-Asn-Asp-Phe-Tyr-His-Glu*-Met-Thr-Asp-Ser-Asn-Val-Ile-Asp-Lys is labeled when the enzyme is inactivated with dicyclohexyl[14C]carbodiimide.

The major radioactive cyanogen bromide peptide derived from the β subunit after inactivating the F$_1$-ATPase from the thermophilic bacterium, PS3 (TF$_i$), with dicyclohexyl[14C]carbodiimide, has been isolated. The amino acid sequences of the COOH-terminal tryptic fragments derived from this cyanogen bromide peptide have been determined. From this analysis it is now known that the amino acid sequence of the β subunit of TF$_i$ in the vicinity of the glutamic acid residue that reacts with dicyclohexylcarbodiimide is Ala-Gly-Val-Gly-Glu-Arg-Thr-Arg-Glu-Gly-Asn-Asp-Leu-Tyr-His-Glu-Met. The hexaresidue segment within this fragment, which is singly underlined, is identical in sequence to that of the labeled peptide obtained from the β subunit of TF$_i$ after it was inactivated with 14C]DCCD (5). The presence of this sequence homology, the non-identity of labeled residues in the carboxyl terminus and the occurrence of significant homology in the amino terminal half of the sequence of the β subunit of EF$_i$ (17) suggests that the carboxyl group of the Glu residues in the carboxyl terminal half of the sequence of the β subunit of EF$_i$ (17) is the site of modification in this enzyme.

The soluble F$_1$-ATPases from a variety of species are inactivated by DCCD in reactions which are slowed in the presence of Mg$^+$ (1-5). Inactivation of TF$_i$, the F$_1$-ATPase from the thermophilic bacterium, PS3, by [14C]DCCD has been correlated with the modification of a specific glutamic acid residue in the β subunit residing in the sequence Ala-Gly-Val-Gly-Glu-Arg where the underlined Glu designates the labeled residue (4). Surprisingly, a different glutamic acid residue was labeled when MF$_i$, the F$_1$-ATPase from bovine heart mitochondria, was inactivated with [14C]DCCD (5). The amino acid sequence of the cyanogen bromide fragment obtained from the β subunit of MF$_i$, which contained the label at the doubly underlined Glu is the following: Glu-Leu-Asn-Asn-Val-Ala-Lys-Ala-His-Gly-Gly-Tyr-Ser-Phe-Ala-Gly-Val-Gly-Glu-Arg-Thr-Arg-Glu-Gly-Asn-Asp-Leu-Tyr-His-Glu-Met. The hexaresidue segment within this fragment, which is singly underlined, is identical to the sequence of the labeled peptide obtained from the β subunit of TF$_i$, after it was inactivated with [14C]DCCD (4). The presence of this sequence homology, the non-identity of labeled residues in the carboxyl terminal half of the sequence of the β subunit of TF$_i$, and the occurrence of significant homology in the amino terminal half of the sequence of the β subunit of EF$_i$ (17) suggests that the carboxyl group of the Glu residues in the carboxyl terminal half of the sequence of the β subunit of EF$_i$ (17) is the site of modification in this enzyme.
FIG. 1. Summary of amino acid sequences around the DCCD-reactive glutamic acid residues of the F1-ATPases. Glu* designates the N-y-glutamyl derivative of dicyclohexyl[14C]urea.

in the two ATPases, and the observed protection against DCCD inactivation provided by Mg2+ to both ATPases, led to the suggestion that the two Glu's highlighted in the bovine sequence shown above might be essential residues in all F1-ATPases. Both glutamic acid residues might function to bind the Mg2+ moiety of magnesium complexes of adenosine nucleotides at the catalytic site. The amino acid sequence analyses presented here for the F1-ATPases from *Escherichia coli*, and the thermophilic bacterium, PS3, support this contention.

EXPERIMENTAL PROCEDURES AND RESULTS

The amino acid sequences around DCCD-reactive carboxyl groups in the subunits of MF1, TF1, and EF1 are shown in Fig. 1. Two positions contain glutamic acid residues which react with [14C]DCCD in different ATPases. One of them is labeled when TF1 is inactivated with [14C]DCCD, while the other is labeled when MF1 and EF1 are inactivated with the radioactive reagent. Besides having a different specificity of labeling with [14C]DCCD, the ATPase from the thermophilic bacterium has two other properties which distinguish it from MF1 and EF1. First, TF1 is unusual in that it exhibits two temperature optima, one at about 23 °C and the other at about 65 °C. (6). Second, the rate of inactivation by DCCD is markedly enhanced in the presence of ADP (4), while the inactivation of MF1 and EF1 is only slightly, if at all, stimulated by ADP (1, 2, 5). The possibility that these factors might influence the specificity of labeling of TF1 by [14C]DCCD has been ruled out by the following experiments. To examine the effect of added ADP on the specificity of labeling, TF1 was inactivated in the presence and absence of 2.0 mM ADP in 50 mM Mes-NaOH, pH 7.8, containing 1 mM EDTA at 23 °C. After removal of excess [14C]DCCD by gel filtration on a Sephadex G-25 column equilibrated and eluted with 0.05 M NH4HCO3, the inactivated protein fractions were denaturated in a boiling water bath and then digested with trypsin known to be contaminated with chymotrypsin at a weight ratio of 0.1 for 20 h at 37 °C. The digests were then subjected to HPLC using the triethylamine-phosphoric acid-acetonic acid elution program described in detail previously (4). The radioactive profiles of the collected fractions were identical, showing a major, sharp radioactive peak appeared at 80 min with some small, less defined radioactive peaks appearing later in the elution profiles. Thus temperature variation and the presence and absence of ADP do not affect the specificity of labeling when TF1 is inactivated with [14C]DCCD.

The results presented show that the radioactivity incorporated into EF1 as a covalent label which survives the purification procedures is associated with the glutamic acid residue marked with an asterisk in Fig. 1. However, the yield of the radioactive tryptic fragment derived from the β subunit on which this assignment is made is surprisingly low when compared to the yields of labeled peptides obtained after inactivating TF1 and MF1 with [14C]DCCD. Extrapolating the values for [14C] incorporation in Table 1 of the miniprint to 100% inactivation, 1.12 g atoms of 14C per mol of EF1; were incorporated which is similar to the value reported by Sare et al. (2) when they inactivated EF1 with [14C]DCCD. On the other hand, TF1 and MF1 were labeled to a greater extent with 2.15- and 2.70-g atoms of 14C per mol having been incorporated into the two enzymes, respectively, after removing excess radioactive reagent by gel filtration under non-denaturing conditions. Structural analyses of TF1 and MF1; after inactivation showed that most of the radioactivity incorporated into these ATPases is due primarily to the reaction of single glutamic acid residue in the β subunit of each with [14C]DCCD (4, 5). While none of the 14C bound initially was removed from TF1 and MF1; during the subsequent isolation of subunits in the presence of 8 M urea, only 45% of the 14C remained bound to EF1, under denaturing conditions. These results suggest that the O-acylsourea formed initially during the inactivation of EF1 rearranges more slowly than do the O-acylsoureas formed initially during the inactivation of TF1 and MF1. If this is indeed the case, then about 50% of the 14C which remains bound to inactivated EF1 after gel filtration under non-denaturing conditions might exist as an O-acylsourea or decomposes on subsequent denaturation. The low incorporation of 14C which is observed during the inactivation of EF1, when compared to that observed during the inactivations of TF1 and MF1; might be due to the attack of the more stable O-acylsourea purporting to be present in EF1, by a neighboring nucleophile. Such a reaction would produce an inactive derivative of the enzyme that would not be radioactive. The mechanism by which O-acylsoureas rearrange to form N-acylureas has not been examined directly. However, the rearrangement of isoimides (21) to the corresponding imides and the rearrangement of S-acylsoureas to N-acylsoureas (22, 23) have been examined in detail. By analogy with these rearrangements, Bruce and his colleagues have suggested that O-acylsoureas rearrange intramolecularly by the attack of the lone pair of the imino nitrogen on the carbonyl carbon, as illustrated in Fig. 2 (22, 24). The favorable geometry for the rearrangement is illustrated with the nucleophilic lone pair cis to the acyl group. Any constraints which would prevent the rearrangement of the O-acylsourea in this conformation would hinder the rearrangement. It is possible that such constraints exist at the

\[ \text{FIG. 2. Schematic representation of the intramolecular rearrangement of the O-acylsourea at the active site of an F1-ATPase (after Pratt and Bruce (22, 23)).} \]
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active site of EF, which prevent the facile rearrangement of the O-$\gamma$-phthalamidacyclobexyl$[^{14}C]$urea derivatives are responsible for the low incorporation of $^{14}C$ that accompanies the inactivation of this ATPase. Satre et al. (2) have suggested that cooperative phenomena might be responsible for the low incorporation of $^{14}C$ when EF$_1$ is inactivated with $[^{14}C]$DCCD. Based on the observation that TF$_1$ and MF$_1$ incorporate much higher amounts of $^{14}C$ specifically, when they are inactivated with $[^{14}C]$DCCD and the arguments presented here, there does not seem to be a strong case to support the suggestion that "less than the sites" phenomena are exhibited when EF$_1$ is inactivated by $[^{14}C]$DCCD (2).

The amino acid sequences shown in Fig. 1 contain invariant glutamic acid residues in the position labeled by $[^{14}C]$DCCD in TF$_1$ and the position labeled by $[^{14}C]$DCCD in MF$_1$ and EF$_1$. Since the rate of inactivation of all three F$_1$-ATPases by DCCD is inhibited by Mg$^{2+}$, it seems appropriate to assume that both carboxyl groups in each ATPase might function to bind the Mg$^{2+}$ moiety of magnesium complexes of adenine nucleotides. However, it must be pointed out that Senior and his colleagues have provided strong evidence which indicates that sites other than the catalytic sites in the F$_1$-ATPases also bind Mg$^{2+}$ (25-27). Moreover, one of these sites, which has high affinity for Mg$^{2+}$, may have a structural role. Therefore, a more detailed analysis will be necessary to determine the function of the glutamic acid side chains which, when modified by DCCD, lead to inactivation of the F$_1$-ATPases.

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EXPERIMENTAL PROCEDURES

General: The ATPases were prepared by previously published procedures (6,7). Incubations with [14C]DCCD were incubated with a phosphate buffer (pH 7.8) and 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS). The ATPase was diluted with 4% (v/v) 32% glycerol (v/v) to a final concentration of 200-1000 pg per ml for the inactivation reaction. The specific activity of the radiolabeled DCCD was determined by titration with unlabeled DCCD in the presence of ATP. The specific activity of [14C]DCCD was determined by equilibrium dialysis and was found to be 6.4 x 10^8 cpm/mmol. The inactivation was carried out by incubating the ATPase with unlabeled DCCD at 0°C for 30 min.

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After the incubation, the samples were separated by SDS-PAGE. The gel was stained with Coomassie Blue and the bands were visualized under UV light. The amount of DCCD in each band was determined by scintillation counting.

The samples were then subjected to liquid chromatography to purify the ATPase. The elution profile of the ATPase from the column is shown in Figure 3.

The ATPase was purified to homogeneity by preparative gel filtration chromatography on a Superose 12 column (Pharmacia). The column was eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 0.002% NaN3.

RESULTS

Location of the Slit 5 in the Sequence Diagram above the F1-ATPase: Figure 4 shows the location of the slit 5 in the sequence diagram above the F1-ATPase. The slit 5 is located at the N-terminal region of the ATPase. The slit 5 is essential for the function of the ATPase, as shown by the observation that the ATPase from a strain carrying a mutation in the slit 5 is inactive.

The results in Table 1 show that the ATPase from a strain carrying a mutation in the slit 5 is inactive. The ATPase from a strain carrying a mutation in the slit 5 is inactive.

Table 1: Amoeba Acid Composition of Peptide Containing r1 Derived from the a-subunit of or F1-ATPase

| Residue | IA | IB | IA + IB |
|---------|----|----|--------|
| Asp     | 6.45 (5) | 5.25 (5) | 11.70 (10) |
| Asn     | 1.00 (1) | 0.69 (1) | 1.69 (2) |
| Gln     | 0.93 (1) | 0.63 (1) | 1.56 (2) |
| Ser     | 2.03 (4) | 2.03 (4) | 4.06 (8) |
| Pro     | 1.05 (1) | 1.05 (1) | 2.10 (2) |
| Ala     | 0.66 (1) | 0.66 (1) | 1.32 (2) |
| Val     | 0.93 (1) | 0.63 (1) | 1.56 (2) |
| Gly     | 0.52 (1) | 0.52 (1) | 1.04 (2) |
| Thr     | 0.68 (1) | 0.68 (1) | 1.36 (2) |
| Ser     | 0.56 (1) | 0.56 (1) | 1.12 (2) |
| Thr     | 0.60 (1) | 0.60 (1) | 1.20 (2) |
| Asp     | 1.00 (1) | 0.60 (1) | 1.60 (2) |
| Asn     | 1.00 (1) | 0.60 (1) | 1.60 (2) |

When the tryptic digest of the eluted, [14C]-labeled a-subunit was subjected to HPLC, the results showed that the peak containing the [14C]-labeled peptide was eluted at 0.5 ml of the 0.2 M KCl fraction as expected. The results also showed that the ATPase from a strain carrying a mutation in the slit 5 is inactive.

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The results in Table 1 show that the ATPase from a strain carrying a mutation in the slit 5 is inactive. The ATPase from a strain carrying a mutation in the slit 5 is inactive.
Figure 5: Resolution of radioactive peptides by chromatography-permeate drops of the "C-labeled tryptic fragment isolated from the z-subunit of TF. The combined chromatography-permeate drops of the peptide in peak B was injected onto the reverse phase HPLC column and then eluted with a linear gradient using 0.1% trifluoroacetic acid for solvent A and 80% acetonitrile for solvent B (v:v). Samples, 1 mL, were collected from the 2 mL fractions for liquid scintillation counting.

The results of this experiment are shown in Table I. The radioactive peptides in each peak were resolved by reversed-phase HPLC and then analyzed by amino acid analysis. The results are presented in Table I. The main peaks, labeled as A and B, were further purified by reverse-phase HPLC and analyzed by amino acid analysis. The results are presented in Table II.

Table I. Amino Acid Analyses of the Peptide Peaks of Figure 4 Containing Tryptic Peptides Derived from the "C-Labeled Fragment Isolated from the ζ-Subunit of TF

| Residue | Peak A | Peak B | Peak C |
|---------|--------|--------|--------|
| Glu     | 5.3    | 12.3   | 115.3  |
| Thr     | 8.5    | 15.8   | 77.3   |
| Ser     | 2.7    | 6.4    | 6.5    |
| Gln     | 13.8   | 4.3    | 6.4    |
| Pro     | 2.0    | 2.0    | 2.7    |
| His     | 0.0    | 2.0    | 4.3    |
| Ala     | 4.5    | 10.4   | 25.5   |
| Val     | 0.0    | 1.0    | 0.0    |
| Leu     | 0.0    | 2.0    | 4.3    |
| Ile     | 4.5    | 10.4   | 25.5   |
| Gly     | 5.3    | 10.4   | 25.5   |
| Arg     | 1.0    | 2.0    | 4.3    |
| Lys     | 4.5    | 10.4   | 25.5   |
| Asp     | 5.3    | 10.4   | 25.5   |
| Glu     | 10.4   | 25.5   |
| Gln     | 10.4   | 25.5   |
| Ser     | 10.4   | 25.5   |
| Thr     | 10.4   | 25.5   |
| Pro     | 10.4   | 25.5   |
| His     | 10.4   | 25.5   |
| Ala     | 10.4   | 25.5   |
| Val     | 10.4   | 25.5   |
| Leu     | 10.4   | 25.5   |
| Ile     | 10.4   | 25.5   |

The amino acid compositions of the major radioactive tryptic peptides derived from the "C-labeled fragment isolated from the ζ-subunit of TF are presented in Table II. The major labeled peptide is Glu-rich and contains significant amounts of Asp, Thr, Ser, Glu, and Lys. The minor labeled peptide is Glu-rich and contains significant amounts of Asp, Thr, Ser, Glu, and Lys. The minor labeled peptide is Glu-rich and contains significant amounts of Asp, Thr, Ser, Glu, and Lys.