Identification of the Dicyclohexylcarbodiimide-binding Subunit of NADH-ubiquinone Oxidoreductase (Complex I)*

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The mitochondrial NADH:ubiquinone oxidoreductase complex (Complex I) is inhibited by N,N′-dicyclohexylcarbodiimide (DCCD), and this inhibition correlates with incorporation of radioactivity from [14C]DCCD into a Complex I subunit of M, 29,000 (Yagi, T. (1987) Biochemistry 26, 2822-2828). Resolution of [14C]DCCD-labeled Complex I in the presence of NaClO4 showed that the labeled M, 29,000 subunit was in the hydrophobic fraction of the enzyme. This fraction, which contains >17 unlike polypeptides, was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the M, 29,000 subunit, containing bound [14C]DCCD, was isolated and purified. The amino acid composition and partial sequence of this subunit corresponded to those predicted from the mitochondrial DNA for the product of the mtDNA gene designated ND-1. The identity of the M, 29,000 subunit with the ND-1 gene product was further confirmed by immunoblotting and immunoprecipitation experiments, using the hydrophobic fraction of [14C]DCCD-labeled Complex I and antiserum to a C-terminus undecapeptide synthesized on the basis of the human mitochondrial ND-1 nucleotide sequence. Thus, it appears that the DCCD-binding subunits of the respiratory chain Complexes I, III, IV, and V in certain organisms the DCCD-binding subunit of the ATP synthase complex (Complex V) are all mtDNA products.

The ATP synthase complexes of mitochondria, chloroplasts, and bacterial membranes, as well as various plasma membrane and vacuolar ATPases, have been shown to be inhibited by the carboxyl group modifying reagent, DCCD (1-7). In addition, it has been demonstrated that proton translocation by the mitochondrial respiratory chain Complexes I, III, IV, and V (ATP synthase) are considered to be so involved.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—Bovine Complex I (12) and the iron-sulfur flavoprotein (FP), the iron-sulfur protein (IP), and the hydrophobic protein (HP) fractions of Complex I (13) were prepared according to the references given.

Purification of Polypeptides from HP—Eighty μg of HP were loaded in each well (1-cm width) of a sodium dodecyl sulfate slab gel (15 x 14 x 0.15 cm) composed of 10% acrylamide as described by Lasemml (14) except that the running buffer contained 0.1 mM thioglycolic acid. The gel was electrophoresed for 3 h at 30 mA and stained for 30 min at room temperature in a solution containing 0.05% Coomassie Brilliant Blue R-250, 25% 2-propanol, and 10% acetic acid. Destaining was carried out for 2-3 h at 4 °C in 5% acetic acid containing 16.5% methanol. The stained bands were sliced from the gel and washed several times with water. The polypeptides were then electroeluted as described by Hunkapiller et al. (15). For amino acid analysis and sequence determination, the isolated polypeptides were re-electrophoresed and re-isolated.

Removal of N-Formyl Group—The amino terminal blocking group of the isolated polypeptide (assumed to be N-formyl) was removed by the modified method of Sheehan and Yang (16) and Fearnley and Walker (17). The purified polypeptide (68 μg) was lyophilized, suspended in 1 ml of 1.5 N HCl in methanol, and incubated for 4 h at room temperature. The solution was neutralized by addition of 1.5
ml of 1 M NaHCO₃, and then concentrated by Centricon-10. The concentrate was washed with 3 ml of 10 mM NaHCO₃ containing 0.02% SDS, lyophilized, and subjected to sequence analysis by a gas phase sequencer (Applied Biosystem).

**Other Analytical Procedures**—Protein was estimated by the method of Lowry et al. (18) or by biuret in the presence of 1 mg of sodium deoxycholate/ml (19). Enzymatic assays were carried out essentially according to Yagi (11, 20). The gels were autoradiographed with skim milk and Tween-20 as blocker (20). Any variations from these procedures and other details are described in the figure legends.

**Materials**—The sources of the chemicals used were as follows: NADH and dithiothreitol were from Behring Diagnostics Inc., Somerville, NJ; DCCD was from Aldrich; [14C]DCCD was from Research Products; acrylamide, SDS, and Coomassie Brilliant Blue R-250 were from Bio-Rad; rotenone was from S. B. Penick; NaClO₂ was from G. Frederick Smith Chemical Co.; and protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. Ubiquinone-1 was a generous gift from Eisai Chemical (Tokyo, Japan). The antiserum to human ND-1 C-terminal undecapeptide was kindly provided by Dr. R. F. Doolittle (University of California, San Diego) and Dr. G. Attardi (California Institute of Technology, Pasadena, CA). Other chemicals were reagent grade or of the highest quality available.

**RESULTS**

**Labeling Pattern of Complex I Polypeptides with [14C]DCCD**—Treatment of Complex I with chaotropic salts results in the resolution of the enzyme complex into a water-soluble and a water-insoluble fraction. The former contains a three-subunit iron-sulfur flavoprotein (FP or the primary NADH dehydrogenase) and a six-subunit iron-sulfur protein (IP). The latter fraction (HP) contains the remainder of the polypeptides of Complex I, phospholipids, and one or two iron-sulfur clusters (4). When this resolution procedure was applied to [14C]DCCD-treated Complex I, protein-bound radioactivity from [14C]DCCD was found exclusively in the HP fraction. These results are shown in Fig. 1. Complex I at 10 mg/ml was incubated with [14C]DCCD until NADH-QI reductase activity was 70% inhibited. The labeled enzyme was precipitated with ammonium sulfate as described in the legend to Fig. 1, resuspended in buffer at 10 mg/ml, and resolved in the presence of 0.5 M NaClO₂, HP, IP, and FP were separated as described and subjected to SDS-gel electrophoresis. A sample of Complex I not treated with DCCD was similarly manipulated and resolved. Fig. 1 shows on the left a protein-stained SDS gel of HP, IP, and FP from [14C]DCCD-treated and control Complex I run side by side. This gel shows that treatment of Complex I with [14C]DCCD does not seem to have affected the resolution of Complex I into HP, IP, and FP, altered the mobility of the polypeptides in these fractions, or created new bands as a result of cross-linking. The right side of Fig. 1 shows an autoradiogram of the gel on the left. It is seen that radioactivity from [14C]DCCD was incorporated only in the polypeptides of the HP fraction. Two polypeptides with Mr values of 49,000 (band 1) and 29,000 (band 2) and several with Mr values <20,000 were labeled. Among these, only the labeling of the M, 29,000 polypeptide correlated with inhibition of NADH-Q₁ reductase activity, as described previously (11). The M, 49,000 band was labeled too rapidly, and the extent of labeling of bands with Mr <20,000 was found to be related neither to the duration of incubation of Complex I with [14C]DCCD nor to the degree of inhibition of NADH-Q₁ reductase activity. Indeed, as will be seen below, these latter bands were not labeled when Complex I at concentrations lower than 10 mg/ml was incubated with [14C]DCCD. This extraneous labeling of low Mr polypeptides may be related to the multiple labeling pattern reported by Voukila and Hassinen (21).

**Isolation of the DCCD-binding Subunit**—In the remainder of this report, the M, 29,000 polypeptide described above will be referred to as the DCCD-binding subunit, because the extent of labeling of only this polypeptide by [14C]DCCD was found to correlate with inhibition of the NADH-Q₁ reductase activity of Complex I (11). Although distinct in the autoradiogram of Fig. 1, the DCCD-binding subunit was found in a region of the SDS gel of HP which contained three closely packed protein bands. These bands are shown in Fig. 2 and will be referred to as the upper, the middle, and the lower bands. Since in this situation autoradiography could not allow one to decide which band was the DCCD-binding subunit, each band was carefully excised from a number of comparable gels. The corresponding slices were combined, protein was

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**Fig. 1. Distribution of radioactivity among the polypeptides of the FP, IP, and HP fractions of [14C]DCCD-treated Complex I.** Complex I at 9.8 mg/ml was incubated with 140 μM [14C]DCCD (1.0 × 10⁵ cpm/mol) in 1 ml of a solution containing 0.25 M sucrose and 50 mM Tris-HCl, pH 7.5, for 2 h at 20 °C. The mixture was diluted with 4 ml of the same solution, and Complex I was precipitated by addition of 2.8 ml of saturated ammonium sulfate solution. The mixture was allowed to stand on ice for 10 min and then was centrifuged for 20 min at 42,500 rpm in a 50 Ti rotor of Beckman ultracentrifuge. The supernatant containing unbound [14C]DCCD was discarded, and the pellet was suspended in 0.8 ml of 50 mM Tris-HCl, pH 8.0, containing 1 mM NaClO₂. NaClO₂ was added from an 8 ml solution to a final concentration of 0.5 M, and the labeled Complex I was resolved and fractionated into HP, IP, and FP according to Galante and Hatefi (13). Protein samples were denatured in the Laemmli sample buffer, containing 80 mM Tris-HCl, pH 6.8, 6% SDS, 0.005% bromphenol blue, and 20% glycerol and were applied to a Laemmli-type 10% acrylamide mini-slab (55 × 95 × 0.75 mm) SDS gel. The amounts of protein applied to the gel were 4 μg each of FP and IP and 12 μg of HP. The gel was electrophoresed for 1 h at 200 V and then stained, destained, and autoradiographed as described previously (11).

**Fig. 2. SDS-polyacrylamide gel of HP stained with Coomassie Blue.** HP (80 μg) was denatured in 15 μl of the Laemmli sample buffer as described in Fig. 1 and loaded on a Laemmli-type SDS slab gel (15 × 14 × 0.15 cm). Electrophoresis, staining, and destaining were as described in Ref. 11.
removed by electroelution and subjected to SDS gel electrophoresis. Results are shown in Fig. 3. The left side of Fig. 3 shows an SDS gel of the isolated upper, middle, and lower polypeptides stained with Coomassie Blue, and the right side shows an autoradiogram of the same gel. It is clear that radioactivity from [14C]DCCD was associated mainly with the lower band. The small amount of radioactivity seen in the middle band is probably due to contamination with the lower band and related to the difficulty in cleanly separating these bands by excision from SDS gels of HP.

**Relationship of the DCCD-binding Subunit to the Mitochondrial Gene Products in Complex I**—The DCCD-binding subunits of Complex III (cytochrome b) and Complex IV (subunit III) are mitochondrial gene products (8, 10, 22). Also, in certain species, e.g. Saccharomyces cerevisiae and possibly maize, the DCCD-binding subunit of the ATP synthase complex is encoded and synthesized within the mitochondria (23). Therefore, it was of interest to see whether the DCCD-binding subunit of Complex I is related to any of the seven Complex I polypeptides which are the products of mitochondrial genes, designated ND-1, -2, -3, -4L, -4, -5, and -6 (24, 25). Among these, the ND-1 gene product was the most likely possibility, because (a) its molecular weight as predicted from the gene (36,000) was closest to that of the DCCD-binding subunit and (b) the human ND-1 gene product with the same predicted molecular weight exhibited an M, of 24,000 on SDS-urea gels (26). Thus, the relationship between the DCCD-binding subunit and the ND-1 gene product was investigated chemically as well as immunochemically. For this purpose, the DCCD-binding subunit (lower band of Figs. 2 and 3) was further purified by repeated SDS-gel electrophoresis and electroelution until no hint of contamination could be detected on stained gels. This material was then subjected to amino acid analysis and N-terminal sequencing. Table I shows the amino acid composition of the DCCD-binding subunit as well as that of the bovine ND-1 gene product as predicted from the gene sequence (22). The similarity of amino acid composition is obvious. The results of the N-terminal sequencing are given in Fig. 4. It is seen that the 15 N-terminal amino acids of the DCCD-binding subunit are identical to those of the bovine ND-1 gene product as predicted from the ND-1 gene sequence.

The immunochemical experiment was carried out as follows. Because antisera to the bovine ND-1 gene product was not available, an antiserum to a synthetic peptide representing the C-terminal undecapeptide of the human ND-1 gene product was employed. This antipeptide antiserum has been shown to cross-react with the bovine ND-1 gene product, because the C terminus of the latter is highly similar to that of its human counterpart (27). As seen in the immunoblot of Fig. 5, the isolated lower band and the same polypeptide in HP cross-reacted with this antipeptide antibody, but the isolated middle and upper bands did not (see Figs. 2 and 3). Other experiments indicated that none of the FP or IP polypeptides cross-reacted with the above antipeptide antibody, either (results not shown).

**Table I**

| Amino acid composition of the DCCD-binding subunit of complex I and the ND-1 gene product as predicted from the gene sequence |
|---------------------------------------------------------------|
| Amino acid | DCCD-binding subunit* | ND-1 gene product |
|------------|-----------------------|-------------------|
|            | mol/mol of DCCD-binding subunit |                 |
| Ala        | 29.1                  | 29                |
| Arg        | 9.2                   | 8                 |
| Asp        | 17.0                  | 15                |
| Cys        | 1                     | 1                 |
| Glu        | 21.7                  | 18                |
| Gln        | 17.2                  | 14                |
| His        | 3.8                   | 4                 |
| Ile        | 25.6                  | 29                |
| Leu        | 54.4                  | 55                |
| Lys        | 8.0                   | 7                 |
| Met        | 18.6                  | 22                |
| Phe        | 17.5                  | 18                |
| Pro        | 22.9                  | 22                |
| Ser        | 20.8                  | 23                |
| Thr        | 18.8                  | 20                |
| Tyr        | 9.0                   | 9                 |
| Val        | 11.1                  | 11                |
| Trp        | 13.1                  | 13                |

The purified DCCD-binding subunit (10 μg) was hydrolyzed in an evacuated and sealed tube with 6 N HCl at 110 °C for 24 h, and amino acid analysis was performed on a Beckman 7300 amino acid analyzer. The values given are averages of three independent analyses. Values under the second column were determined from the gene sequence. Values under the first column were calculated on the basis of the total number of amino acids of the ND-1 gene product, excluding cysteine and tryptophan; —, not determined.

**Fig. 4.** Comparison of the N-terminal amino acid sequence of the purified DCCD-binding subunit with that predicted from the DNA sequence of bovine ND-1 gene. Procedures for removal of the N-formyl group of the DCCD-binding subunit and sequence analysis are described under “Experimental Procedures.”

The amino acid yield at each cycle was as follows: 1, Met (186 pmol); 2, Phe (120 pmol); 3, Met (131 pmol); 4, Ile (110 pmol); 5, Asn (92 pmol); 6, Ile (92 pmol); 7, Leu (97 pmol); 8, Met (110 pmol); 9, Leu (90 pmol); 10, Ile (76 pmol); 11, Ile (87 pmol); 12, Pro (62 pmol); 13, Ile (96 pmol); 14, Leu (44 pmol); 15, Leu (60 pmol).
The DCCD-binding Subunit of Complex I

**FIG. 5.** Immunoblot analysis of HP and the isolated upper (U), middle (M), and lower (L) bands (see Fig. 3) with antiserum to a synthetic C-terminal undecapeptide of the human ND-1 gene product. Proteins were electrically transferred from SDS-polyacrylamide gels to nitrocellulose membranes (Schleicher and Schuell, 0.22-µm pore size) as described by Towbin et al. (31). The nitrocellulose membranes were incubated with 1% skim milk serum to a synthetic C-terminal undecapeptide of the human ND-1 gene product for 105 min at 37 °C and then with antiserum (1:500 dilution) to the C-terminal undecapeptide of the human ND-1 gene product overnight at 4 °C and then with anti-rabbit antibody conjugated to horseradish peroxidase (1:100 dilution) for 45 min at 37 °C. The nitrocellulose membranes were transferred into substrate solution which contained in the above phosphate-NaCl buffer 0.36 mg/ml of 4-chloronaphthol and 0.012% 3,3′-diaminobenzidine (Anti ND-IC). Thus, the results of Figs. 3–6 establish that inactivation of Complex I by DCCD is associated with modification of a single polypeptide of M, 29,000 (36,000 as predicted from the bovine gene sequence) and that this polypeptide is the product of the mtDNA gene, ND-1.

**FIG. 6.** Immunoprecipitation of the DCCD-binding subunit with antiserum to the C-terminal undecapeptide of the human ND-1 gene product (Anti ND-1C). Immunoprecipitation experiments with the antipeptide antiserum were carried out according to Anderson and Blobel (32). [3H]DCCD-labeled HP (1.93 mg/ml) was incubated for 2 h at 30 °C in a buffer containing 0.25 M sucrose, 50 mM Tris-acetate, pH 7.5, and 4.5% SDS. Fifty µl of the HP solution was diluted with 200 µl of a buffer containing 190 mM NaCl, 60 mM Tris-Cl, pH 7.5, 6 mM EDTA, and 1.25% Triton X-100 and then incubated overnight at 4 °C with 10 µl of the antipeptide antiserum or control serum. The suspensions were centrifuged for 2 min in an Eppendorf centrifuge. The supernatants were transferred to new Eppendorf tubes, 60 µl of 1:1 suspension of protein A-Sepharose CL-4B in the dilution buffer were added to each supernatant, and the mixtures were incubated with end-over-end mixing for 2 h at room temperature. The resins were washed three times with 1 ml of a buffer containing 145 mM NaCl, 50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.1% Triton X-100, and 0.02% SDS and then washed once with the same buffer lacking detergent. Forty µl of Laemmli's sample buffer (see Fig. 1) was added to each tube and incubated for 2 h at 30 °C. After centrifugation for 30 s, the supernatants (8 and 15 µl) were subjected to SDS-gel electrophoresis and autoradiography as described in Fig. 1. The heavily stained bands near the top of the gels are IgG.

**FIG. 7.** Effect of rotenone on incorporation of [14C]DCCD into the DCCD-binding subunit. Complex I at 1 mg/ml was incubated for 2 h at 20 °C with 90 µM [14C]DCCD in the presence of rotenone (0 µg/ml, lane 3; 2.2 µg/ml, lane 4; 6.5 µg/ml, lane 5; 19.4 µg/ml, lane 6; and 58.8 µg/ml, lane 7). The extents of inhibition of NADH-Q reduc tase activity of Complex I at the above rotenone concentrations were, respectively, 0, 73, 96, 98, and 100%. Lane 2 received Complex I incubated for 2 h at 20 °C and then treated with 90 µM [14C]DCCD at 0 °C and immediately denaturated in the Laemmli sample buffer (Fig. 1). Marker proteins in lane 1 were bovine serum albumin (M, 66,000), ovalbumin (M, 45,000), glyceraldehyde-3-phosphate dehydrogenase (M, 36,000), carbonic anhydrase (M, 29,000), trypsinogen (M, 24,000), trypsin inhibitor (M, 20,100), and α-lactalbumin (M, 14,200). Lanes 2–7 contained 11 µg each of Complex I. For other details, see Fig. 1.

**DISCUSSION**

Two polypeptides of apparent M, 49,000 and 29,000 are labeled when bovine Complex I is treated with [14C]DCCD. The former is labeled rapidly and saturated when inhibition of NADH-Q reductase activity of Complex I reaches about 50%. However, the labeling of the M, 29,000 polypeptide parallels the inhibition of NADH-Q reductase activity by [14C]DCCD and reaches saturation only when this activity is completely inhibited (11). These results indicated, therefore,
that the M, 29,000 polypeptide was involved in the NADH-ubiquinone reductase activity of Complex I and contained an essential carboxyl group whose modification by DCCD resulted in inhibition of ubiquinone reduction (11, 30). Voukila and Hassinen (21) have reported that treatment of Complex I at 37 °C with [14C]DCCD results in the labeling of six subunits with molecular masses of 13.7, 16.1, 21.5, 39, 43, and 53 kDa. First of all, this extensive labeling may be related to the incubation temperature used, because incubation of Complex I at 37 °C results in structural alterations and loss of rotenone-sensitive Q reductase activity (11, 20). Second, as it was indicated above, we also observed the labeling of additional polypeptides when Complex I at high concentration (e.g. 10 mg/ml) was incubated with [14C]DCCD. However, the extent of labeling of these additional polypeptides appeared to be unrelated to the duration of incubation of Complex I with [14C]DCCD and to the degree of inhibition of NADH-Q1 reductase activity. Considering the fact that Complex I contains a large number of hydrophobic polypeptides (4), such spurious labeling by [14C]DCCD, especially under adverse conditions, is not surprising. Voukila and Hassinen (21) do not report on the correlation between inhibition of NADH-ubiquinone reductase activity and the extent of labeling by [14C]DCCD of the several polypeptides indicated above. However, in our experiments, such a correlation was observed with respect to labeling of only the M, 29,000 polypeptide. Thus, it was considered justified to designate this polypeptide as the DCCD-binding subunit of Complex I.

Resolution of [14C]DCCD-treated Complex I by chaotropic salts and separation of the water-soluble FP and IP fragments from the water-insoluble HP fraction showed that the DCCD-binding subunit was in HP, not in FP and IP which contain most of the electron carriers of Complex I. The DCCD-binding subunit was excised from SDS gels of HP, purified by repeated gel electrophoresis and electroelution, and subjected to amino acid analysis and N-terminal sequencing down to the 15th residue. These data showed excellent agreement with the predicted amino acid composition and N-terminal sequence of the ND-1 gene product of mitochondrial DNA. Furthermore, the antiserum to a synthetic peptide corresponding to the C-terminal undecapeptide of the human ND-1 gene product cross-reacted with the isolated DCCD-binding subunit of Complex I and immunoprecipitated this subunit labeled with [14C]DCCD from the HP fraction of [14C]DCCD-treated Complex I. The similarity of the C termini of the human and bovine ND-1 gene products is known (22), and the above antipeptide antiserum had been shown earlier to cross-react with the corresponding mtDNA gene product of bovine mitochondria (27, 29). Thus, the results reported here establish that the DCCD-binding subunit of Complex I is the product of the bovine mtDNA gene designated as ND-1.

The finding that the DCCD-binding subunit of Complex I is a mitochondrial gene product is interesting in view of the fact that the DCCD-binding subunits of Complex III (cytochrome b) and Complex IV (subunit III) are also mtDNA products. In addition, the DCCD-binding subunit of the ATP synthase complex in S. cerevisiae and possibly maize is mtDNA-encoded as well. It might also be added that in Neurospora the gene for the DCCD-binding subunit of the ATP synthase complex is present in both the mitochondrial and the nuclear DNA, but only the latter is expressed (23).

In terms of the effect of DCCD, the above energy-transducing systems may be divided into two groups. In Complexes III and IV, DCCD modification appears to disengage the scalar and the vectorial reactions, because DCCD inhibits proton translocation much more than electron transfer (8, 10). In the ATP synthase complex, this disengagement does not occur. As a result, ATP hydrolysis is inhibited when proton translocation through the F0 sector of the enzyme complex is blocked by DCCD. In Complex I, electron transfer and proton translocation are also blocked in parallel by DCCD (11). This could mean that the primary effect of DCCD is on electron transfer, which necessarily inhibits proton translocation as well. Should this be the case, then the DCCD-binding subunit of Complex I may be involved in electron transfer. This is entirely possible, because iron-sulfur center N-2 of Complex I, which has a relatively high reduction potential and is considered to be the immediate electron donor to ubiquinone, appears to fractionate into HP. The polypeptide bearing iron-sulfur center N-2 would be expected to be hydrophobic, because it would have to interact with the water-insoluble ubiquinone-10. It could also be the site of rotenone binding, because this reagent inhibits electron transfer immediately on the substrate side of ubiquinone.

Another interpretation of concomitant inhibition by DCCD of electron transfer and proton translocation by Complex I would be that DCCD acts on Complex I as it does on the ATP synthase complex. It blocks proton translocation by Complex I without disengaging the proton channel from the subunits involved in electron transfer. Consequently, electron transfer becomes inhibited to the same extent that proton translocation is blocked by DCCD. Should this interpretation be correct, then another analogy between Complex I and the ATP synthase complex may be considered. In the ATP synthase complex, the catalytic sector (F1) is water-soluble, whereas the sector involved in proton translocation through the membrane (F0) is composed of hydrophobic polypeptides. In Complex I, also, the IP (which is concerned with NADH oxidation) and the FP are water-soluble and contain FMN and six of the eight iron-sulfur clusters of Complex I, whereas HP (which most likely contains the polypeptides that form the proton channel of Complex I) is composed of hydrophobic polypeptides and contains the DCCD-binding subunit of the enzyme complex. The fact that several energy-transducing NADH-ubiquinone reductases that have been examined from prokaryotic and eukaryotic sources are all inhibited by DCCD, whereas those not containing an energy-coupling site are not (11), could be a hint in favor of the latter possibility.

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