Sordarin derivatives are selective inhibitors of fungal protein synthesis, which specifically impair elongation factor 2 (EF-2) function. We have studied the effect of sordarin on the ribosome-dependent GTPase activity of EF-2 from Candida albicans in the absence of any other component of the translation system. The effect of sordarin turned out to be dependent both on the ratio of ribosomes to EF-2 and on the nature of the ribosomes. When the amount of EF-2 exceeded that of ribosomes sordarin inhibited the GTPase activity following an inverted bell-shaped dose-response curve, whereas when EF-2 and ribosomes were in equimolar concentrations sordarin yielded a typical sigmoidal dose-dependent inhibition. However, when ricin-treated ribosomes were used, sordarin stimulated the hydrolysis of GTP. These results were compared with those obtained with fusidic acid, showing that both drugs act in a different manner. All these data are consistent with sordarin blocking the elongation cycle at the initial steps of translocation, prior to GTP hydrolysis. In agreement with this conclusion, sordarin prevented the formation of peptidyl-[3H]puromycin on polysomes from Candida albicans.

Sordarin is a natural product originally isolated from broths of Sordaria araneosa (1) (see chemical structure in Fig. 1). The potential of sordarin derivatives as new antifungal drugs of promoting therapeutic value has been evidenced recently. Their spectrum includes most fungal pathogens that affect immunocompromised patients, i.e. Candida spp., Cryptococcus neoformans, Pneumocystis carinii, and several endemic American fungi as well as dermatophytes (2). These compounds exert their antifungal activity by selectively inhibiting the elongation step of protein synthesis in the target organisms, but not in higher eukaryotes (3).

Protein synthesis is an essential process common to all living cells. It is composed of three phases, namely initiation, elongation, and termination. The machinery involved in the process includes mRNA, tRNAs, ribosomes, and several soluble non-ribosomal protein factors. It has been shown recently that elongation factor 2 (EF-2) is the only component of the whole system that is able to bind sordarin; although EF-2 itself can bind the drug, high affinity binding requires the presence of ribosomes (4). On the other hand, mutations either in EF-2 or in the ribosomal protein P0 may confer resistance to sordarins (5–7). This evidence has led to EF-2 being considered as the primary sordarin-binding protein and the EF-2-ribosome complex as the functional target.

By interacting with the ribosome, EF-2 promotes translocation and catalyzes the hydrolysis of GTP. Translocation is a crucial step in the elongation cycle of protein synthesis. Briefly, it consists of the simultaneous movement of the newly formed peptidyl-tRNA and the recently decacylated tRNA from the ribosomal A site to the P site and from the P site to the E site, respectively, while the ribosome moves ahead one codon on the mRNA. It proceeds through a complicated mechanism where several events take place in a coordinate and precise fashion to maintain translational accuracy. Translocation has been thoroughly studied in prokaryotes, and there are several models attempting to explain the process (see Refs. 8 and 9 for recent reviews), but it is still far from being completely understood. Nevertheless, it is generally accepted that the ribosome oscillates between a pre- and a posttranslational state, which refers to two small scale, but significantly different, conformational arrangements within the particle. Both states are separated by a high activation energy barrier of 90 kJ/mol, which is reduced by EF-2 in eukaryotes or EF-G in prokaryotes in the direction from PRE to POST (10, 11). The role of GTP hydrolysis in this scenario is not clearly defined. For many years, it was believed that GTP acted as a modulator of the affinity of the elongation factor for the ribosome, as described for G-proteins (12). Accordingly, the EF-2-GTP complex would have high affinity for the PRE ribosome, whereas the EF-2-GDP complex would have low affinity for the POST ribosome. Translocation would thus precede GTP hydrolysis, and the resulting GDP molecule would trigger the release of EF-2 from the ribosome (13). However, it has been postulated recently that GTP hydrolysis occurs before translocation, providing the energy needed for the process (14). Consequently, EF-2 would behave as a mechanical protein more than as a G-protein. Nevertheless, this interpretation is still a matter of debate and has not yet been fully accepted (15, 16).

In the present paper we study the effect of sordarin on GTP hydrolysis catalyzed by EF-2 and ribosomes from the pathogenic yeast Candida albicans, in the absence of any other elements of the protein synthesis machinery (i.e. the so-called “uncoupled GTPase”). The results obtained have allowed us to propose a model that may clarify the mode of action of sordarin and validate this family of compounds, not only as therapeutic agents, but also as useful tools for studying the molecular mechanisms underlying protein synthesis.

**EXPERIMENTAL PROCEDURES**

Elongation factor 2, salt-washed ribosomes, and polysomes were isolated from C. albicans 2005E as described previously (4). EF-2 concentration was determined spectrophotometrically using $E_{280}$ as deduced from its amino acid sequence. Ribosome concentration was also calculated spectrophotometrically assuming one $A_260$ unit corresponds to 18 pmol of ribosomes (17). Sordarin was provided by the Bioprocessing Unit at Glaxo Wellcome R & D (Stevenage, United Kingdom). [γ-32P]GTP (370 MBq/mL, >150 MBq/nmol) was from Amer...
sordarin in whole-cell and in cell-free protein synthesis is a response curve. Sordarin caused some inhibition at intermediate concentrations (0.1 µM) but this effect disappeared either at lower or higher levels of the drug. This peculiar behavior can be interpreted as a combination of two processes. As the effect of sordarin, while the low affinity/high catalysis process (due to PRE) is inhibited by high affinity/low catalysis process (due to POST) is stimulated by the drug. It should be noted that the ribosome preparation used in this assay is a heterogeneous mixture of PRE and POST ribosomes, but according to previous results, the PRE population outnumbers the POST by 4 to 1 (19–21). Consequently, in the absence of any inhibitor, the PRE population shows high affinity for the complex than POST ribosomes. The presence of sordarin would lead to the formation of a nonproductive PRE-EF-2-GTP-sordarin complex, thus explaining the descending half of the curve. On the other hand, the excess of EF-2 present in the assay medium may undergo drolysis (in the absence of EF-2 and ribosomes) were subtracted from each determination provided their values never exceeded 5% of the overall. Likewise, GTP hydrolysis due to EF-2 alone and to ribosomes alone were measured and discounted for each sample. For ricin treatment, ribosomes were preincubated for 15 min at 37 °C in the presence of ricin (molar ratio ricin/ribosomes = 10/1) and then used as explained above.

Puromycin Reaction—Freshly prepared polysomes from C. albicans were tested for their ability to synthesize peptide-[3H]puromycin according to (18). Samples containing 5 A260 units of polysomes were incubated with the corresponding drug for 15 min at 30 °C in the presence of 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl2, 10 mM 2-mercaptoethanol, 500 µM [γ-32P]GTP (3.5 kBq), 0.5 µM ribosomes, 0.5 µM or 2 µM EF-2 and the corresponding amount of compound tested. Samples were preincubated at room temperature for 15 min with the corresponding drug in the absence of GTP; GTP was added, and mixtures were incubated at 37 °C during 30 min. The released [32P]phosphate was extracted as phosphomolybdate with a cyclohexane/isobutanol, 1:1 (v/v) mixture and the radioactivity measured. Blanks corresponding to spontaneous GTP hydrolysis (in the absence of EF-2 and ribosomes) were subtracted from each determination provided their values never exceeded 5% of the overall. GTPase activity in systems with excess of EF-2 over ribosomes. The presence of sordarin would lead to the formation of a nonproductive PRE-EF-2-GTP-sordarin complex, thus explaining the descending half of the curve. On the other hand, the excess of EF-2 present in the assay medium may undergo drolysis (in the absence of EF-2 and ribosomes) were subtracted from each determination provided their values never exceeded 5% of the overall. Likewise, GTP hydrolysis due to EF-2 alone and to ribosomes alone were measured and discounted for each sample. For ricin treatment, ribosomes were preincubated for 15 min at 37 °C in the presence of ricin (molar ratio ricin/ribosomes = 10/1) and then used as explained above.

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The maximum stimulatory effect is reached at 1.0 μM sordarin, whereas fusidic acid inhibited the reaction as in the previous experiment. It is interesting to observe that the maximum stimulatory effect of sordarin on the uncoupled GTPase activity of equimolar mixtures of EF-2 and ribosomes is reached at 1.0 μM sordarin, the same concentration at which maximum recovery of activity was observed in Fig. 2.

Results shown in Figs. 3 and 4 are in agreement with the assumptions made above. It can also be clearly seen in Fig. 4 that sordarin is not a direct inhibitor of the GTPase activity. Hence, the inhibition observed in Fig. 3 (and partially in Fig. 2) must be considered more as a consequence than as a primary effect. Previous data on the binding of sordarin to EF-2ribosome complexes (4) make it unlikely that sordarin prevents the interaction between PRE ribosomes (the majority) and EF-2-GTP. It follows that sordarin may directly impair the transition from PRE to POST, thus blocking the ribosome in the pretranslational state.

To test this conclusion we studied the effect of sordarin on the so-called puromycin reaction (18, 24) and compared it to that of cycloheximide and fusidic acid. This is a classical tool for exploring the mode of action of protein synthesis inhibitors. It is based on the different accessibility of the ribosomal A site to radiolabeled puromycin depending on whether the ribosome is in PRE (i.e., A site inaccessible to puromycin) or in POST (A site accessible).

Actively translating polysomes were isolated from C. albi- cans. In the absence of any other drug, [3H]puromycin was incorporated to the nascent peptide chains (Fig. 5). This same result was obtained when polysomes were previously incubated with fusidic acid, as expected (25). However, a significant decrease in the incorporation of [3H]puromycin was observed when polysomes were preincubated with sordarin, a result similar to that obtained with cycloheximide. Anisomycin, a known inhibitor of peptide bond formation (20), also reduced the amount of peptidyl-[3H]puromycin formed. The effect of sordarin was clearly dose-dependent as deduced from Fig. 6, although it was not possible to reach total inhibition of the reaction. This fact is explained by the spontaneous translocation undergone by a fraction of the polysomes during the iso-
translation procedure previous to incubation with the inhibitor. Consequently, the mode of action of sordarin seems to be closer to that of cycloheximide (inhibition of the translocation step (20)) than to that of fusidic acid (inhibition of the turnover of EF-2z GDP from the POST ribosome (26)).

**DISCUSSION**

The unexpected shape of the dose-response curve obtained when studying the effect of sordarin on the uncoupled GTPase reaction catalyzed by *C. albicans* EF-2 may give a clue to the precise mode of action of the drug. The curve has been interpreted on the basis of the two reciprocating conformations of the ribosome and has been considered a consequence of the experimental conditions used in the assay, mainly the large excess of EF-2 over ribosomes and the heterogeneous nature of the ribosomal preparation, which is a mixture of PRE and POST particles. Changing these conditions gives rise to a complete change of the curve shape. From a qualitative point of view, the sordarin curve in Fig. 2 can be considered a combination of the corresponding curves in Figs. 3 and 4, in agreement with the interpretation that the former result is due to a combination of two processes. A full cycle describing the molecular events taking place in vitro in the uncoupled GTPase assay is depicted in Fig. 7, an adaptation of the model described by Nygård and Nilsson (19). According to our results, sordarin is not a direct inhibitor of the GTPase activity, since the drug is able to stimulate GTP hydrolysis under certain conditions. Inhibition of this activity, when observed, seems to be a consequence of blocking the elongation cycle upstream of GTP hydrolysis. There are two ways to inhibit the cycle upstream of GTP hydrolysis: by preventing the interaction between PRE and EF-2z GDP or by blocking the transition from PRE-EF-2-GTP to POST-EF-2-GTP. The first possibility is considered unlikely in the light of previous results showing high affinity binding between sordarin and the ribosome-EF-2 complex. The evidence points therefore to the transition from PRE-EF-2-GTP to POST-EF-2-GTP as the protein synthesis step inhibited by sordarin.

In the previous paragraphs we have interpreted our results according to the classical model of translocation suggested by Nygård and Nilsson (19) 10 years ago. Presently more detailed models have been proposed. All of them are based on experimental data obtained with bacteria systems. However there...
are no apparent reasons not to apply them to eukaryotic systems. The first one is the “hybrid states” model proposed by Moazed and Noller (27), which has evolved into the “elaborated hybrid states” model (8). The latter incorporates the hypothesis formulated by Rodnina et al. (14, 28) that places GTP hydrolysis before translocation and considers GTP the source of energy that allows the mechanical movement of tRNAs and mRNA. The tRNAs move in alternate fashion with respect to ribosomal subunits, thus yielding the designated hybrid states: the acceptor arms of the tRNAs are in the E or P sites of the large ribosomal subunit, while the anticodon arms remain in the P or A sites of the small subunit, respectively. One of the main implications of this model is that the structural rearrangements that take place in the ribosome are subtle local changes rather than gross conformational changes. The model is composed of several intermediate states that are generated in the following sequence: 1) generation of a puromycin-unreactive hybrid state on the pretranslocated ribosome immediately after peptide bond formation, 2) binding of EF-2-GTP, 3) transition to a puromycin-reactive hybrid state, 4) GTP hydrolysis, 5) translocation of the anticodon arms, and 6) release of EF-2-GDP. This sequence of events is compatible with the proposed scheme in Fig. 7. Step 3 may be the one that shifts the PRE conformation of the ribosome into the POST. The subsequent events may exclusively affect tRNAs, mRNA and EF-2-GTP. Accordingly, step 3 would be the one targeted by sordarin.

The alternative model is the so-called alpha-epsilon model, proposed by Nierhaus and co-workers (29). This model suggests the existence of a movable ribosomal domain (called “α-ε”), which shifts from the A and P sites to the E and P sites during translocation, carrying with it the tRNAs. The precise role of EF-2 and GTP hydrolysis is not clearly assigned, but by analogy to the previous model, it seems reasonable to propose that the ultimate effect of sordarin may be blocking the movement of the domain.

Recently, Justice et al. (5) have suggested that sordarins stabilize the POST-EF-2-GDP complex in a manner identical to fusidic acid. This conclusion was based on the observation that sordarin hampered the exchange between unlabeled GTP and \(^{3}H\)GTP previously bound to EF-2 and ribosomes from S. cerevisiae, the ribosomes being in 7-fold excess over the factor. Our results, however, show a completely dissimilar behavior between sordarin and fusidic acid. The result reported by Justice et al. (5) may be explained by the experimental conditions used. A 7-fold excess of ribosomes over EF-2 would lead to most EF-2 molecules being bound by the high affinity PRE ribosomes, which would be locked into a nonproductive PRE-EF-2-GTP complex by sordarins, according to the model in Fig. 7.

Sordarins have proved to be a family of drugs of potential medical and scientific value. Their therapeutic efficacy, their unpaired specificity, and their novel mode of action in the field of antifungals justify their interest. However, the molecular details that account for sordarin activity still remain elusive as the precise events of translocation do. More studies orientated to elucidate the former will also be useful to understand the latter.

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