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Electron Spin Resonance Evidence for a Free Radical Intermediate in the Cis-Trans Isomerization of Furylfuramide by Oxygen-Sensitive Nitroreductases

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SUMMARY

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Tatsumi *et al.* proposed that the enzymatic cis-trans[1] isomerization of furylfuramide is the result of anion free radical formation by nitroreductases. Electron spin resonance measurements of the furylfuramide anion free radical have provided spectral evidence of this intermediate, and clarified the disputed relationship between the isomerization and the nitro reduction of furylfuramide.

INTRODUCTION

Furylfuramide (AF-2)[2] has an extensive literature on its mutagenicity, carcinogenicity and cytotoxicity (1, 2). Enzymatic nitro reduction of AF-2 is thought to form the reactive metabolites which are primarily responsible for these effects. The synthetic form of this former food additive is the cis isomer. Typical mammalian nitroreductases, such as xanthine oxidase or rat liver microsomal nitroreductase, isomerize cis-AF-2 to trans-AF-2 before they initiate reductive "activation" (3). Many investigators have observed this isomerization in the course of investigations of the reductive activation of cis-AF-2 by mammalian (3-5) and bacterial systems (6, 7). Tatsumi *et al.* (4) have proposed that this enzymatic cis-trans isomerization is a direct consequence of enzymatic nitro reduction via the following mechanism (Fig. 1). The nitroreductases, which are inhibited by oxygen, transfer a single electron to nitro substrates to give their respective anion free radicals (8-12). The carbon-carbon double bond linking the furan rings of AF-2 would be weakened by anion radical formation, because the additional electron is in an antibonding molecular orbital. Upon formation the cis-AF-2 anion free radical was proposed to quickly isomerize to the trans-AF-2 anion, which

[1] Cis-trans refers to the relative position of the furan rings attached to the olefinic double bond, i.e., either the same or opposite sides, respectively.

[2] Abbreviations: AF-2, furylfuramide or 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; ESR, electron spin resonance.

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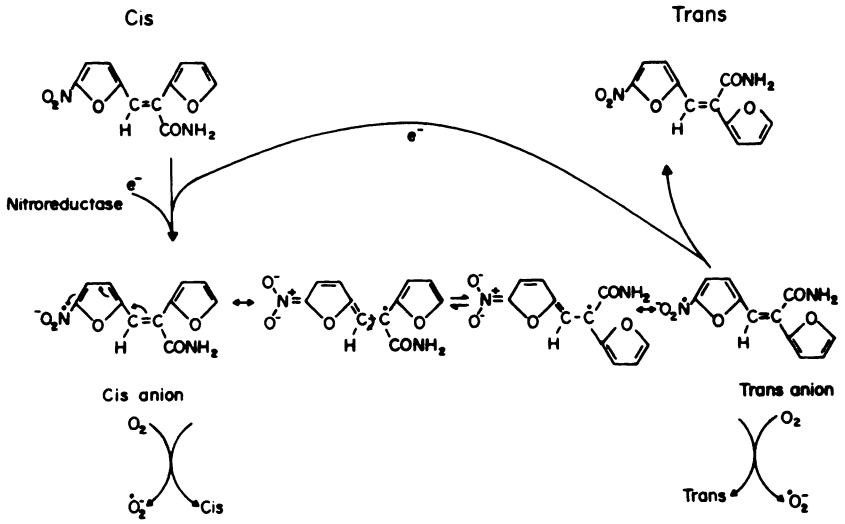

FIG. 1. *Postulated mechanism for the cis-trans isomerization of AF-2 by oxygen-sensitive nitroreductases, as modified from Tatsumi et al. (4).*

could then be oxidized to form trans-AF-2 (Fig. 1). Tatsumi *et al.* showed that a purified nitroreductase, butter milk xanthine oxidase, would catalyze the isomerization of cis-AF-2 to trans-AF-2 as well as the reverse reaction and that the nitro group was necessary for this activity, but provided no other evidence in support of their isomerization mechanism.

Recently, Tomoeda and Kitamura studied the isomerization and nitro reduction of cis-AF-2 by subcellular fractions of *E. coli* (7). Their observations suggested that the nitro reducing and isomerizing activities of *E. coli* were due to totally different enzymes, in apparent contradiction of the isomerization mechanism of Tatsumi *et al.* (4). We chose to investigate the nitro reduction of cis- and trans-AF-2 with ESR, because this technique can distinguish the cis-trans conformers of the proposed free radical intermediate and can, under certain conditions, give the rate of isomerization. Our ESR studies have clarified the nature of the relationship between the isomerization and the nitro reduction of AF-2.

MATERIALS AND METHODS

Cis-AF-2 was a gift from Taijiro Matsushima of the Institute of Medical Science, the University of Tokyo (m.p. 154–155°; literature m.p. 151–152° (4)). Trans-AF-2 was prepared by heating cis-AF-2 in 33 mM phosphate buffer (pH 7.0) at 90° for 2.5 hr (5). Recrystallization from benzene gave red crystals (m.p. 174–176°; literature m.p. 176–177° (4)). Rat hepatic microsomes were prepared and the protein concentration was determined as described previously (10). ESR spectra of anaerobic microsomal incubations at 25° were obtained with a Varian century series E-109 spectrometer equipped with a TM_{110} cavity. Visible spectra of cis- and trans-AF-2 were obtained with a DW-2A Aminco-Chance spectrophotometer at 37°.

RESULTS

The ESR spectrum of an anaerobic microsomal incubation containing AF-2 and a NADPH-generating system provides direct evidence of free radical formation (Fig. 2A). In the presence of air, nitroaromatic anion free radicals undergo rapid air oxidation to form superoxide anion, and the nitroaromatic anion free radicals are not detected (8–12). In addition, identical spectra were obtained with either cis- or trans-AF-2. These ESR spectra of the free radical intermediates did not vary with time even though visible spectroscopy shows a rapid and nearly complete conversion of cis-AF-2 to trans-AF-2 under the same conditions (Fig. 3).

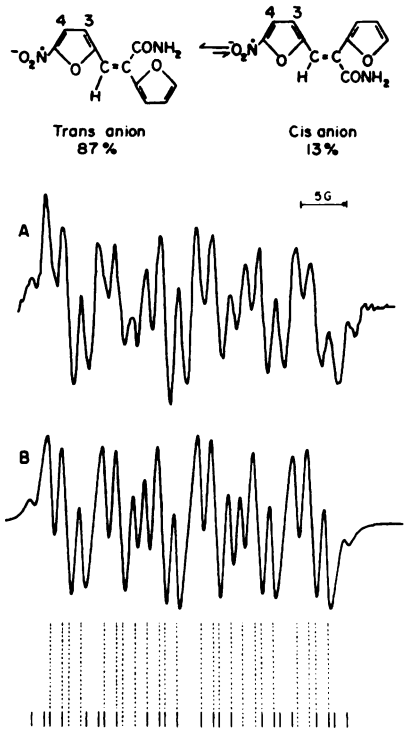

FIG. 2. *A. The first-derivative ESR spectrum of a mixture of trans- and cis-AF-2 anion free radicals observed on anaerobic incubation of 0.5 mM cis-AF-2 (or trans-AF-2) with a NADPH generating system (0.67 units/ml glucose-6-phosphate dehydrogenase, 5.5 mM glucose-6-phosphate and 0.39 mM NADP) and 1 mg/ml of hepatic microsomal protein from male rats in KCl-Tris-$MgCl_2$ (150 mM, 50 mM, 5 mM pH = 7.4) buffer*

The AF-2 was first dissolved in ethylene glycol monomethyl ether (0.4% by volume). B. A computer simulated ESR spectrum of a mixture of cis- and trans-AF-2 anion radicals in the ratio of 13:87. The hyperfine coupling constants were as follows: trans-AF-2 ($a^N_{NO_2}$ = 10.2 G, a_4^H = 5.75 G, a_3^H = 1.9 G & a^H_{vinyl} = 1.3 G) and cis-AF-2 ($a^N_{NO_2}$ = 12.2 G, a_4^H = 5.75 G, a_3^H = 1.9 G & a^H_{vinyl} = 1.3 G). The stick diagram demonstrates that the two outer lines are due to cis-AF-2 radical anion alone, whereas all other lines are a composite of both cis (——) and trans (- - -) anion free radical components.

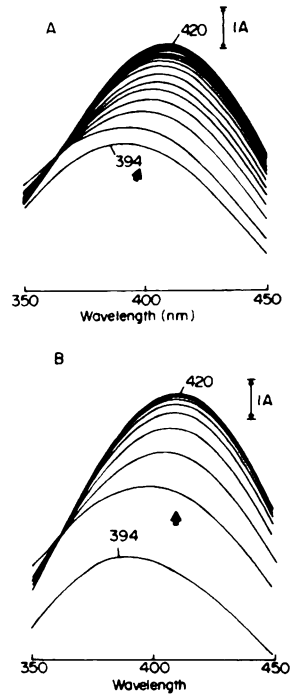

FIG. 3. *A. Change in visible absorption curves during the microsomal isomerization of cis-AF-2 to trans-AF-2*

Incubation contained: 0.67 units/ml glucose-6-phosphate dehydrogenase, 5.5 mM glucose-6-phosphate, 0.13 mM NADP, 0.5 mg/ml microsomal protein, and 50 μM cis-AF-2 in KCl-tris-$MgCl_2$ (150 mM, 50 mM, 5 mM, pH = 7.4) buffer. After preincubation for 5 min the reaction was initiated with microsomes and scanned at 10 nm/sec. AF-2 was omitted from the reference cuvette. B. The contents of the sample and reference cuvettes were the same as above, except the sample cuvette was purged with nitrogen and scanned at 20 nm/sec.

Computer simulation of the spectrum represented by all but the outer two lines shown in Fig. 2A reveals that the radical contains a nitrogen atom and three hydrogens. The hyperfine couplings of this predominant species show excellent agreement with the previously reported parameters of the nitrogen and the ring protons for other nitrofuran compounds (9). In addition, a hydrogen coupling apparently due to the hydrogen attached to one of the vinyl carbons is also observed. With a decreased magnetic field modulation of 0.2 gauss, each line of the central region of the spectrum in Fig. 2 can be partially resolved to show many hyperfine couplings of about 0.3 gauss. These couplings presumably arise from the more distant nuclei of the second furan ring and the amide group. With the higher magnetic field modulation of 0.67 gauss, these small hyperfine couplings are

completely unresolved, thus accounting for the 0.9 gauss peak-to-peak Lorentzian line width used in the computer simulation (Fig. 2B).

The less abundant species represented by the two outer lines of the composite spectrum shows no detectable g-value shift from the predominant species, implying that it is also a nitrofuran anion free radical. The spectrum of the less abundant species is largely obscured and, for the purposes of computer simulation, we have assumed that the ESR parameters of the two free radicals differ only in their nitrogen hyperfine coupling constant. This assumption was made because the nitrogen coupling of nitrofuran anions is highly variable, and a small increase in this coupling is all that is necessary to accomodate the greater width of the less intense spectra. The best computer simulation assumed a 13:87 ratio for the concentration of the two nitrofuran anion free radicals. This ratio was determined from amplified and expanded spectra of the first two lines shown in Fig. 2A. Computer simulations also showed that the composite spectrum is largely insensitive to changes in the hydrogen hyperfine couplings of the less abundant free radical, as long as the position of the outer two lines remains unchanged.

Although spectral evidence alone indicates that both the major and the minor components of the spectrum represent AF-2 anion free radicals, the assignment of the spectrum to cis-trans anion radicals must be made on other grounds. Preliminary INDO (intermediate neglect of differential overlap) molecular orbital calculations on cis- and trans-AF-2 anions suggest that the cis anion radical will have the larger nitrogen hyperfine splitting constant (calculation not shown). Furthermore, the greater steric hindrance expected for the cis-conformer should result in a predominance of the trans-conformer. In the parent compounds, the greater thermodynamic stability of trans-AF-2 is clearly indicated by the relative conversion of cis-AF-2 to trans-AF-2 (87–91%) *vs* that of trans-AF-2 to cis-AF-2 (7–11%) observed in xanthine oxidase incubations under optimum conditions (4). Clearly, since xanthine oxidase is only a catalyst, and cannot change the thermodynamic equilibrium of cis- to trans-AF-2, the equilibrium ratio of cis to trans is approximately 10:90. The similarity in the equilibrium ratios of cis- to trans-AF-2 and the two free radicals is the strongest chemical evidence for our assignment of the two species as the respective anion radicals. The observation of distinct conformational isomers with ESR only requires that the equilibrium mixture be slowly interconverting on the ESR time scale, which is very rapid. More precisely, the lifetime of the conformers must be much longer than $1/\gamma_e (a^N_{cis} - a^N_{trans}) = 28$ nanoseconds. This reaction can still be fast in terms of chemical reaction, and in fact, competes favorably with the autoxidation of the AF-2 anion free radicals (Fig. 3), although the air inhibition of the isomerization is greater than fifty percent. Assuming that the rate of cis-AF-2 anion oxidation is equal to its rate of isomerization, an estimation of the half life of the cis anion conformer can be made.

$$V_{iso} = V_{oxid}$$
$$= k_{oxid}[O_2][\text{cis-AF-2 anion}]$$
$$= k_{iso}[\text{cis-AF-2 anion}]$$

From these equations k_{iso} is seen to be approximately equal to $k_{oxid}[O_2]$. Assuming k_{oxid} is 2×10^5 $M^{-1}s^{-1}$ (10) and $[O_2]$ is approximately 200 μM, k_{iso} is calculated to be 40 s^{-1}, which is equivalent to a cis anion half life of 17 msec. This rapid interconversion of the cis-trans anion conformers is in contrast to the geometric isomers of the parent compounds, which require hours at elevated temperatures to obtain equilibrium. From the ratio of the ESR signal amplitudes the equilibrium constant, K = [trans-AF-2 anion]/[cis-AF-2 anion], can be calculated as 87/13 or 6.7. From the equilibrium constant the difference in the Gibbs free energy for the two anion radical conformers, or the conformational free energy, can be calculated as 1.1 kcal/mole.

DISCUSSION

We have presented spectral evidence that the cis-trans isomerization of AF-2 pro-

ceeds via an equilibrium mixture of cis and trans radical anions, which occur at approximately the same ratio as the equilibrium mixture of the parent isomers. The formation of the anion radical appears to be essential for the cis-trans conversion as proposed by Tatsumi *et al.* and illustrated in Fig. 1. Tomoeda and Kitamura made three observations which suggested that the nitro reducing and isomerizing activities of *E. coli* toward AF-2 were due to different enzymes. Briefly, their first observation was that the isomerizing activity was observed at lower concentrations of pyridine nucleotides than that required for reductase activity. Secondly, the isomerizing activity is dicumerol insensitive, whereas the reducing activity was inhibited by dicumerol. The third observation was made using nitrofuran-resistant mutants. They found that whereas the resistant bacteria had lost a substantial amount of reductase activity toward AF-2, the mutation did not induce the loss of isomerizing activity. The reconciliation of these observations with the isomerization mechanism of Tatsumi *et al.* is based upon the fact that *E. coli* contains two different nitroreducing activities, distinguishable primarily by their sensitivity to oxygen. The oxygen-insensitive reductase has a high activity in the wild-type *E. coli*, but is absent from nitrofuran-resistant mutants (13, 14). The observations of Tomoeda and Kitamura were made in the presence of air, therefore this reduction of AF-2 is of the oxygen-insensitive type. *E. coli* contains a second type of nitroreductase, which, like most mammalian nitroreductases, is oxygen-sensitive. This activity is present in both the wild type and the mutant strain of *E. coli*. The results of Tomoeda and Kitamura and the isomerization mechanism of Tatsumi *et al.* are not contradictory if the oxygen-insensitive *E. coli* reducing activity does not form the anion radical intermediate, and the oxygen-sensitive *E. coli* reducing activity does form the anion radical, as has been recently demonstrated (15). In the present study we provide electron spin resonance evidence for cis- and trans-AF-2 anion radical intermediate formation during the microsomal cis-trans isomerization of AF-2. This study further elaborates the earlier view that the nitro radical anion is an obligatory intermediate of nitro reduction by oxygen-sensitive nitroreductases (8, 9).

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