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AN ELECTRON SPIN RESONANCE INVESTIGATION AND MOLECULAR ORBITAL CALCULATION OF THE ANION RADICAL INTERMEDIATE IN THE ENZYMATIC *CIS-TRANS* ISOMERIZATION OF FURYLFURAMIDE, A NITROFURAN DERIVATIVE OF ETHYLENE

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The enzymatic *cis-trans* isomerization of nitrofuran derivatives has been proposed to occur via the formation of a radical anion intermediate. ESR investigations, in conjunction with intermediate neglect of differential overlap (INDO) molecular orbital calculations, support this concept by demonstrating the enzymatic generation of *cis* and *trans* radical anions of 3-(5-nitro-2-furyl)-2-(2-furyl) acrylamide. The INDO calculations further indicate that the rotational barrier between the *cis* and *trans* anion radicals of this compound is only 5–10 kcal/mol, whereas a 70 kcal/mol barrier exists for the parent geometric isomers. Hyperfine splitting constants for the *cis-trans* conformers have been assigned on the basis of INDO calculations. Surprisingly, only the nitrogen hyperfine splitting of the nitro group is distinguishably different in the two conformers, a result which is not inconsistent with the INDO calculations.

Introduction

Enzyme catalyzed *cis-trans* isomerization about carbon-carbon double bonds is a relatively unstudied area [1]. The isomerization of *cis*-3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide (AF-2) to *trans*-AF-2 by a variety of enzymes which reduce nitro compounds has recently received considerable attention [1–4]. *Cis*-AF-2, also known as furylfuramide, was used as an antibacterial food additive in Japan until it was found to be carcinogenic, mutagenic and cytotoxic [5,6]. These biological activities of AF-2 and of other nitro compounds are thought to be initiated by enzymatic nitroreduction. Several investigators have observed that the isomerization of *cis*-AF-2 precedes nitro-reduction [1,2,4]. Tatsumi et al. [1,2] have proposed that this *cis-trans* isomerization is a direct consequence of nitro anion radical formation by nitro-reducing enzymes (Fig. 1).

In fact, the carbon-carbon double bond linking the furan rings of AF-2 would be weakened to some extent by anion radical formation, because the additional electron is in an antibonding molecular orbital. The weakened double bond is represented by the valence-bond structure I in Fig. 1. Such a species can clearly rotate about the former double bond to give valence-bond structure II. The loss of an electron by the *trans* conformer to an electron acceptor such as O_2 or another molecule of *cis*-AF-2 results in the formation of *trans*-AF-2.

The nitro group is necessary for the observation of isomerization by the nitro-reducing enzyme, xanthine oxidase [1]. Many other purified nitro-reducing enzymes, as well as reductive radiolysis, will support isomerization of AF-2 and closely related compounds [2]. Further, inferential biochemical support for the anion radical isomerization mechanism is found in the fact that *Escherichia coli* contain nitro-reducing enzymes which neither form an anion radical intermediate nor catalyze the isomerization of AF-2 [4].

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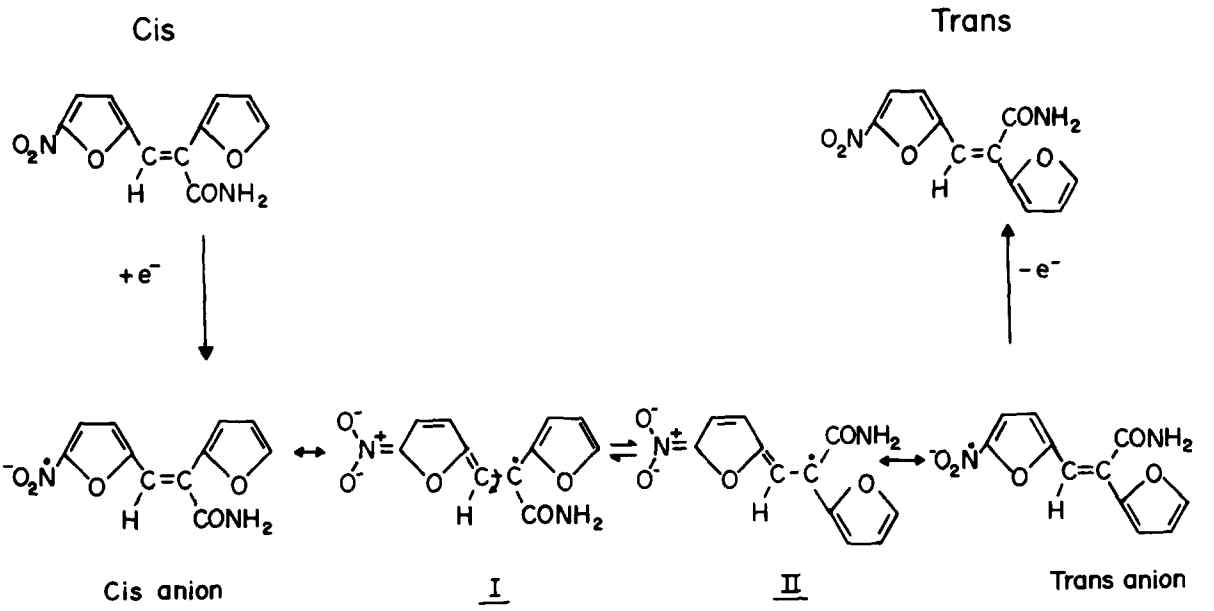


Fig. 1. Postulated mechanism for the *cis-trans* isomerization of AF-2 by nitro-reducing enzymes or other one-electron donators [1].

In addition, the presence of interconverting *cis*- and *trans*-AF-2 anion free radical intermediates has been observed with ESR in microsomal incubations [4], which contain the one-electron donating, nitro-reducing enzyme, NADPH-cytochrome *c* reductase [7]. In the present work, better resolved ESR spectra are assigned using a combination of partial deuteration and intermediate neglect of differnetial overlap (INDO) molecular orbital calculations. In addition, the INDO calculations further indicate the feasibility of the anion radical mechanism by showing that the barrier to rotation about the carbon-carbon double bond is decreased by anion radical formation from 70 kcal/mol to only 5–10 kcal/mol. This effect on the barrier to rotation about the carbon-carbon double bond is not obvious, because this bond is distant from the nitro group where most of the negative charge and spin density of nitro anion radicals is well known to reside.

Experimental section

Cis-AF-2 was a gift from Taijiro Matsushima (Institute of Medical Science, University of Tokyo). The *cis* or *trans* form means the isomer in which the furan rings lie is on the same or opposite sides of the ethylene bond, respectively. All biochemicals were obtained from Sigma.

Rat hepatic microsomes were prepared and the protein concentration was determined as described previously [4], except for those incubations where the buffer was made with ^2H_2O. In these cases, the microsomes were washed once in buffer made with ^2H_2O.

ESR spectra of anaerobic microsomal incubations at 25°C in 150 mM KCl/50 mM Tris-HCl_5/5 mM $MgCl_2$ (pH 7.6) buffer were obtained with a Varian century series E-109 spectrometer equipped with a TM_{110} cavity. The large nitrogen hyperfine couplings were obtained from the simulation in Fig. 3. The other splittings were obtained from the simulations shown in Fig. 4, where the condition $a^{2H}_{ND_2} = (\gamma^2H_2/\gamma H)a^{H}_{NH_2} = 0.1$ G was used.

INDO calculations. All bond-length, angle, and π-bond order calculations were carried out with an INDO program, QCPE Number 141, adapted for use on a Univac 1110 system. Standard lengths and angles were used for the furan and the nitrofuran as well as

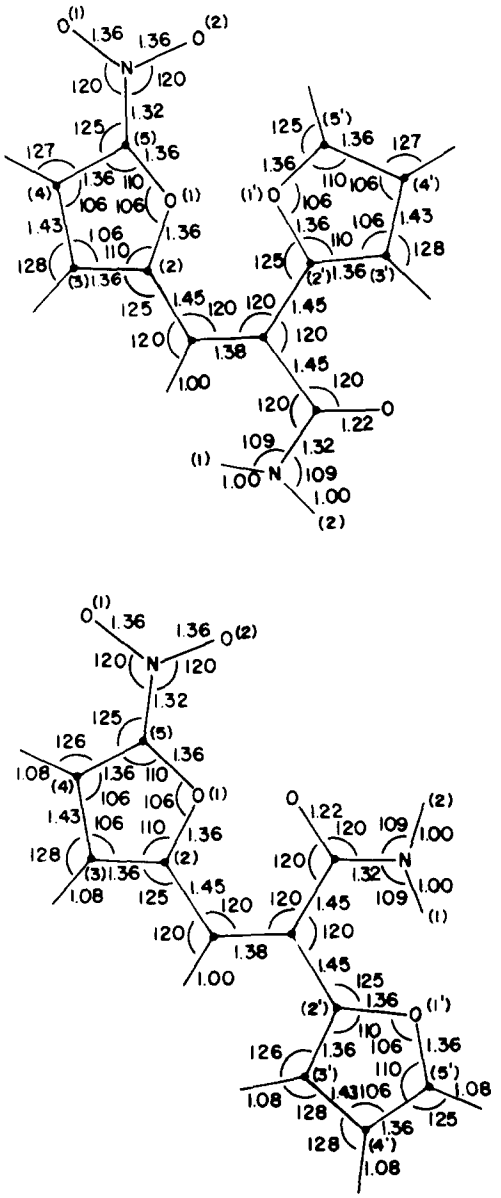


Fig. 2. The geometrical parameters for the *cis*- and *trans*-AF-2 anion radicals in their minimum energy configurations.

the amide moiety [8]. Only bonds to the ethylene carbons were minimized. The nitro-furyl group was assumed to be planar, because previous INDO calculations [9–11] and single crystal ESR investigations [12] have shown that neither pyramidal nor twist distortion of the nitro group in the nitrobenzene anion radical is important. Convergence of the INDO calculations was, in general, not good (to within 0.0005 Hartree), but perhaps not unexpected in view of the size of the molecule. As a result efforts to minimize the bond angles about the ethylene carbons were not carried out.

The effect of rotation of anion about the olefinic bond was explored for a limited number of conformers defined by the dihedral angle χ including *cis*-AF-2 ($\chi = 0°$) and *trans*-AF-2 ($\chi = 180°$). The energy minimum for the *cis* conformer occurs at $\chi = 20°$ and for the *trans* conformer at $\chi = 170°$. The bond angles and lengths for these two configurations are shown in Fig. 2.

Results and Discussion

The room temperature hyperfine splitting constants at the nitrogen and at the various hydrogen atoms for *cis*- and *trans*-AF-2 anion radical are reported in Table I. These hyperfine couplings are used in the simulations shown in Fig. 3 and 4 of the ESR spectra obtained from the anaerobic microsomal incubations containing *cis*-AF-2 and a NADPH-generating system.

Previous investigations had shown that the ESR spectra obtained were the same whether *cis*- or *trans*-AF-2 was used, and that the hyperfine couplings and the relative line amplitudes did not change over several minutes. Simulation of a spectrum similar to the one in Fig. 3 (but in H_2O buffer) indicated that two substituted-nitrofuran radicals were present, and that these radicals differed mainly in their relative populations and in their nitrogen hyperfine splitting constants. The two species have indistinguishable *g*-values, as is consistent with the radicals being structurally closely related.

The experimental spectra shown in Fig. 3 and Fig. 4A were obtained from incubations in which the buffers were made with ^2H_2O. Apparently, the hydrogen atoms on the amide group are exchangeable and have significant hyperfine couplings. This resulted in a spectrum which is better resolved in the wings, and a corresponding inprovement in the reported hyperfine splitting constants (Table I), and in the relative population of the observed radicals (Fig. 3). The four largest hyperfine coupling constants are as much as 0.4 G different from the earlier determination [4].

TABLE I

CALCULATED AND EXPERIMENTAL HYPERFINE COUPLING CONSTANTS (G) OF *TRANS* AND *CIS* 2-(2-FURYL-3-(5-NITRO-2-FURYL) ACRYLAMIDE ANION RADICALS

Nucleus and position	*Trans*-AF-2		*Cis*-AF-2	
	Theor.	Exptl. *	Theor.	Exptl.
$a^{N}_{NO_2}$	6.13	10.4	7.31	12.1
$a^{H}_{C(3)H}$	3.23	2.0	3.78	2.0
$a^{H}_{C(4)H}$	−3.58	5.3_5	−4.54	5.3_5
$a^{(H)}_{C=(H)}$	2.38	1.6	2.47	1.6
$a^{N}_{NH_2}$	0.49	0.3_5	−0.27	0.3_5
$a^{H(1)}_{NH_2}$	0.29	0.6_5	0.12	0.6_5
$a^{H(2)}_{NH_2}$	0.16	0.6_5	0.01	0.6_5
$a^{H}_{C(3')H}$	−2.33	0.3_5	−2.29	0.3_5
$a^{H}_{C(4')H}$	0.97	<0.2	0.79	<0.2
$a^{H}_{C(5')H}$	−1.62	<0.2	−1.41	<0.2
$a^{(C)}_{C=(H)}$	−425.85	–	−421.09	–

* Estimated error is ±0.1 G for *trans*-AF-2 hyperfine coupling constants. The *cis*-AF-2 hyperfine coupling constants are assumed values as described in the text.

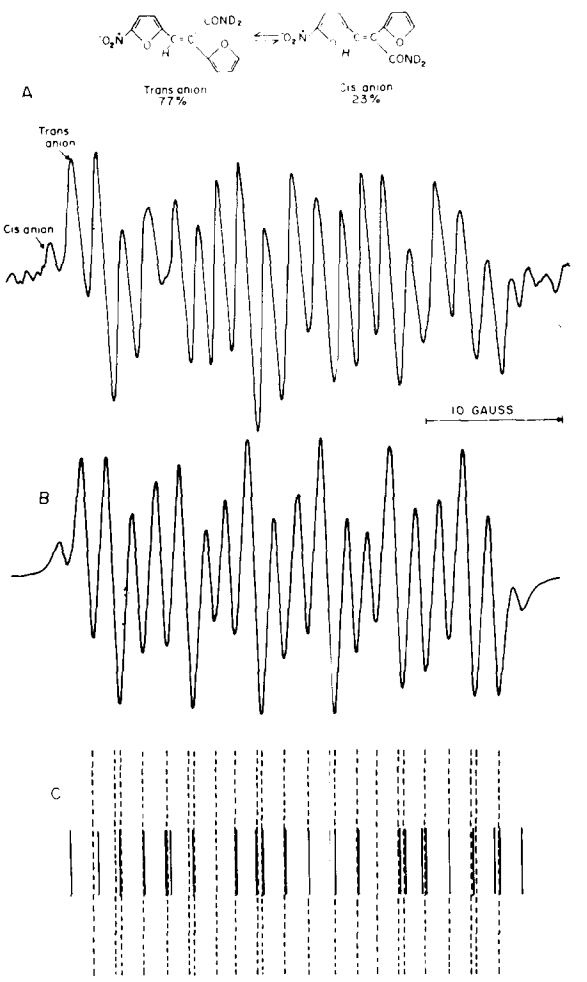


Fig. 3. A. The ESR spectrum of a mixture of *trans*- and *cis*-AF-2 anion free radicals observed on anaerobic incubation of 2.0 mM *cis*-AF-2 with a NADPH-generating system (1.3 units/ml glucose-6-phosphate dehydrogenase, 11 mM glucose 6-phosphate and 0.8 mM $NADP^+$) and 4 mg/ml hepatic microsomal protein from male rats in H_2O-based buffer. The AF2 was first dissolved in ethylene glycol monomethyl ether (1.6%, v/v). The modulation amplitude was 0.67 G and the nominal microwave power was 5 mW. B. A computer simulated ESR spectrum of a mixture of *trans*- and *cis*-AF-2 anion radicals in the ratio of 77 : 23. The hyperfine coupling constants in Table I which are greater than 1.0 G were used in the simulation with a Lorentzian line width of 0.8 G. C. 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.

With lower modulation amplitude and microwave power, the center of the spectrum observed in buffers made with H_2O can be resolved (Fig. 4C), and provides another test of the accuracy of the hyperfine coupling constants. This part of the spectrum corresponds to $M_I = 0$ for $a^{N}_{NO_2}$. The absence of corresponding resolution for $M_I = \pm 1$ probably arises from two factors. First, nitro anion free radicals are commonly characterized by line width variations with significant contributions of the line width proportional to M_I^2, as is better known for nitroxide free radicals. Second, if $a^{N}_{NO_2}$ is different for the *cis* and *trans* conformers, but the other hyperfine splitting

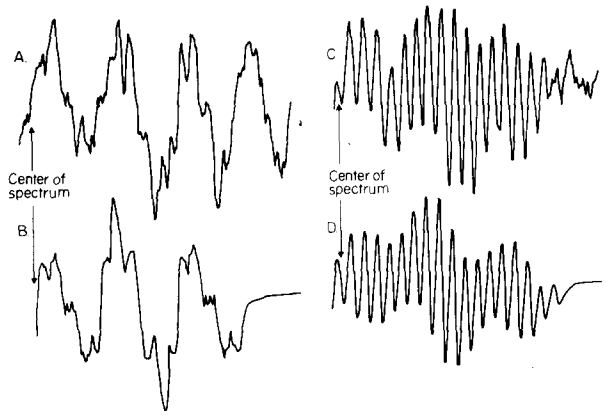


Fig. 4. A. The ESR spectrum of the first four lines to the right of the center of the spectrum in Fig. 3A. The microsomal incubation is the same as that described in Fig. 3. The modulation amplitude was decreased to 0.1 G. B. A computer-simulated ESR spectrum using all of the hyperfine couplings shown in Table I except $a^{H}_{NH_2} = 0.6_5$ G which has been replaced by a $a^{H}_{ND_2} = 0.1$ G. Only those lines with $M_I = 0$ for $a^{N}_{NO_2}$ have been used in the simulation, therefore, the fourth line in Fig. 4A which arises from $M_I = -1$ is not simulated. A Lorentzian line width of 0.13 G was used. C. The ESR spectrum of the same region shown in Fig. 4A. All experimental conditions are the same except that the buffer was made with H_2O instead of ^2H_2O. Under these conditions, the unresolved fourth line seen clearly in Fig. 4A is undermodulated and is not distinguishable from the noise. D. A computer-simulated ESR spectrum using all the hyperfine couplings shown in Table I, with $M_I = 0$ for $a^{N}_{NO_2}$ with a Lorentzian line width of 0.2 G.

constants are nearly equal, then the region where $M_I = 0$ will be better resolved as indicated by the stick diagram in Fig. 3.

With the assumption that the hyperfine couplings of *cis*- and *trans*-AF-2 are the same except for $a^{N}_{NO_2}$ and that the reduction in the number of resolved lines upon substituting ^2H_2O for H_2O in the buffer is due to the exchange of the two hydrogen atoms of the amide group, the simulations in ^2H_2O-buffer (Fig. 4B) and H_2O-buffer (Fig. 4D) were obtained. The assignment of the two $a^{N}_{NO_2}$ hyperfine splitting constants is made primarily on the basis of the INDO calculations, which show that *cis*-$a^{N}_{NO_2}$ is greater than *trans*-$a^{N}_{NO_2}$. The experimental difference (*cis*-$a^{N}_{NO_2}$ – *trans*-$a^{N}_{NO_2}$) = 1.7 G is in good agreement with the theoretical 1.2 G. The assignment of the larger $a^{N}_{NO_2}$ to the *cis* conformer is also consistent with the lower expected relative abundance of this radical due to its increased steric hindrance. The assignment of $a^{H}_{C(4)H}$ to the largest hydrogen hyperfine splitting is based on INDO calculations (Table I) as well as the results of methyl substitution on the nitrofuran anion radical [10]. The difference in the calculated $a^{H}_{C(4)H}$ for the two conformers is comparable to the difference between the experimental and theoretical $a^{H}_{C(4)H}$. With these two exceptions, the INDO calculations indicate that the two conformers have very similar electron distributions as might be expected for conformational isomers which differ primarily in their steric hindrance. The assignment of $a^{H}_{C(3)H}$ and $a^{H}_{C=H}$ is based solely on the INDO calculations. The nitrogen and ring hydrogen hyperfine couplings are in good agreement with the previously reported parameters of related nitrofuran anion radicals [13,14].

The assignment of $a^{H}_{NH_2}$ is based on the isotope substitution observed in ^2H_2O buffer. Only one hyperfine coupling must be assigned to the second furan ring, whereas the INDO calculations indicate that all three of the hydrogen atoms of the furan ring will have observable hyperfine couplings. This disagreement with the INDO calculations could probably be resolved by twisting this furan ring about the bond linking it to the olefinic carbon, thereby decreasing its conjugation with the rest of the aromatic system.

The theoretical hyperfine coupling constants of the low natural abundance nuclei were unexceptional, except for the carbon hyperfine coupling for the olefinic carbon linked to the nitrofuryl group (Table I). The most striking feature of this calculation is this unusually large, negative *s* orbital spin density. As the *cis* conformer is rotated into the *trans* conformer, the spin density on this carbon remains less than –0.5 and parallels the total energy of the anion radical (Fig. 5). Due to an insufficient signal-to-noise ratio (the natural abundance of ^{13}C is only 1.1%), we have not searched for this most unusual C^{13} satellite.

From the limited number of rotational conformers calculated there appear to be energy minima at about $\chi = 20°$ and $\chi = 170°$ (Fig. 5). That this should be the case is not surprising in view of the crowding problems of the furan moieties. The fact that the energy minimum for the *cis* conformer occurs at a dihedral angle of 20°, whereas the *trans* minimum occurs at a dihedral angle of only 10° is not surprising

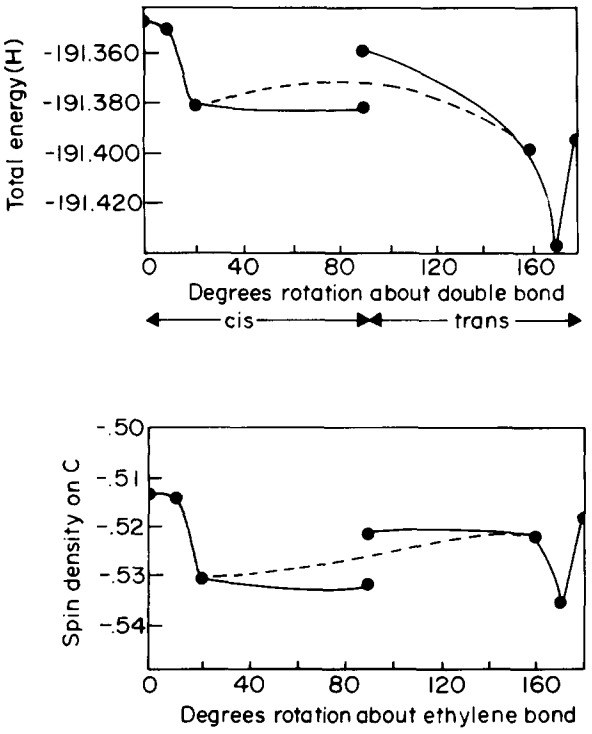
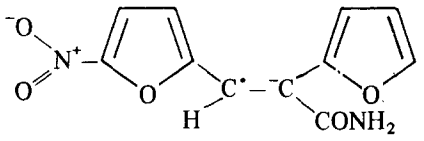


Fig. 5. INDO-computed potential energy for the rotation about the ethylene bond of the anion radical of AF-2 (upper). The corresponding INDO-computed spin density on the olefinic carbon bound to the nitrofuryl group (lower). The 90° calculation failed to converge. The two points shown near 90° are approaches to 90° from the *cis* and *trans* conformations.

inasmuch as the crowding of the two furan moieties in the *cis* isomer is much more severe and would require a larger dihedral angle to relieve.

The calculation of spin and charge densities (Fig. 6) in *cis*- and *trans*-AF-2 anion radicals gives an indication of the appropriateness of the valence-bond structures which have been used to rationalize the rotation of the anion radical about the carbon-carbon double bond. Both valence-bond structure III [1,2], which is formed by the addition of the odd electron to the ethene linkage,



III (*cis* or *trans*)

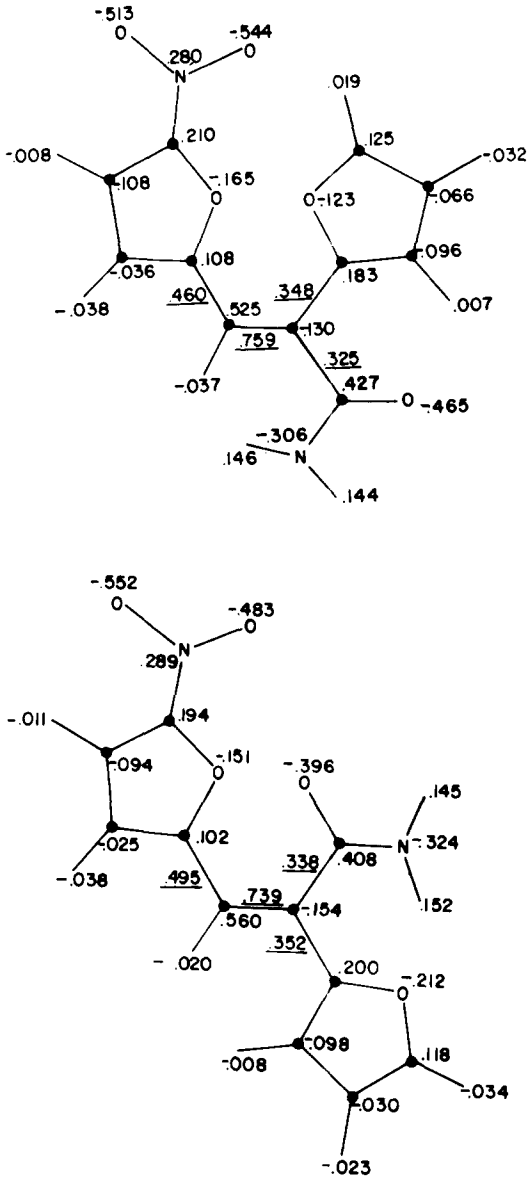
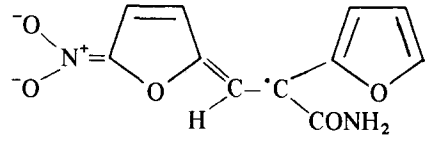


Fig. 6. INDO-computed charge density for *cis*- and *trans*-AF-2 in their minimum energy configurations. The π-bond order of the carbon bonds to the olefinic carbons are underlined.

and IV (Fig. 1)



IV (*cis* or *trans*)

have been used to indicate the single-bond character of the bond between the ethylene carbons in AF-2 anion radicals. The first valence-bond structure predicts a large positive p_z orbital spin density on the ethylene carbon attached to the nitrofuryl group, whereas a negative spin density was calculated (−0.10 for *trans* and −0.15 for *cis*). In contrast, valence-bond structure IV correctly shows a large positive p_z orbital spin density on the other ethylene carbon (0.19 for *trans* and 0.21 for *cis*). In addition, the second valence-bond structure correctly shows most of the negative charge residing on the oxygens of the nitro group and positive charge on the nitrogen (Fig. 6). Valence-bond structure IV appears to be a more reasonable rationalization of the single-bond character of this nominal double bond.

The thermodynamic stability of molecular *trans*-AF-2 is clearly indicated by the relative conversion of *cis*-AF-2 to *trans*-AF-2 (≃86%) vs. that of *trans*-AF-2 to *cis*-AF-2 (≃10%) observed for several nitroreducing enzymes [2]. Since enzymes are only catalysts and cannot change the thermodynamic equilibrium of *cis*- to *trans*-AF-2, the equilibrium ratio of *trans*- to *cis*-AF-2 is approx. 86 : 10. A similar *trans* to *cis* ratio of 77 : 23 is found for the AF-2 anion radicals (Fig. 1). The population ratio or equilibrium constant between either the parent molecules or their anion radical conformers is equivalent to a difference in the Gibbs free energy of approx. 1 kcal/mol. Our INDO calculations correctly indicate that molecular *trans*-AF-2 will be more stable than *cis*-AF-2, but the calculated energy difference of approx. 32 kcal/mol is too large.

Although the thermodynamic ratio of *cis* to *trans*, or the conformation free energy, is similar for both the anion radical and the parent AF-2, the barrier to rotation is much lower for the anion radical. Whereas hours at elevated temperatures are required to obtain equilibrium of the parent AF-2, the half-time for isomerization of the *cis* anion radical has been estimated at only 17 ms [4]. Our INDO calculations are in accord with these observations in that the energy difference between the parent *cis*-AF-2 and the 90° twisted conformation is 70 kcal/mol, whereas the corresponding rotatational barrier for the anion radical is only 5–10 kcal/mol. Although the π-bond order between the ethylene carbons of the *cis*- and *trans*-AF-2 conformers perhaps appears large, it is not much different from the π-bond orders of the neighboring nominally single bonds (Fig. 6).

At a typical steady-state concentration of 2 μM, the nitro anion radical chemical half-life due to disproportionation has been estimated to be approx. 1 min [15], therefore the rapid isomerization will maintain the interconversion equilibrium between *cis* and *trans* conformers regardless of differences in the much slower rates of decay or formation of the *cis* or *trans* conformers. Note the same composite spectrum is obtained when either *cis*- or *trans*-AF-2 is reduced enzymatically [4].

It should be noted that very closely related nitrophenylethylene derivatives form *cis* and *trans* anion radicals which do not interconvert for at least several minutes [15,16]. Recently it was reported that *cis*-3-(4-nitrophenyl)-2-(2-furyl) acrylamide does not isomerize in the presence of nitro-reducing enzymes [17], which further supports the role of the anion radical in enzymatic isomerization. This further indicates that anion-free radical formation does not necessarily form rapidly interconverting *cis-trans* conformers in even closely related molecules. The conversion of *cis*-stilbene radical anion to *trans*-stilbene radical anion can actually occur most rapidly through the formation of the *cis*-stilbene dianion and isomerization of the latter, because isomerization of *cis*-stilbene anion radical is generally slow [18]. Clearly not all radical anions containing an ethene bond undergo rapid isomerization. However, for the system investigated here, rapid *cis-trans* conversion of the radical ion does appear to occur. For this system, ESR and INDO investigations have helped to establish the feasibility of the anion radical mechanism of AF-2 isomerization by confirming the identity of the *cis*- and *trans*-AF-2 anion radicals and by showing that the anion does indeed have a lower rotational barrier and ethene bond order than the molecular AF-2.

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