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Excited Indole-3-aldehyde from the Peroxidase-Catalyzed Aerobic Oxidation of Indole-3-acetic Acid. Reaction with and Energy Transfer to Transfer Ribonucleic Acid†

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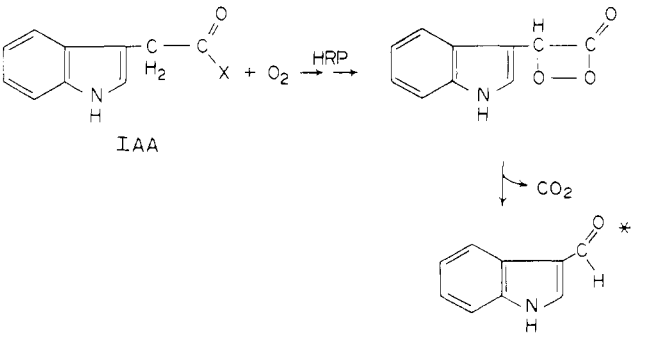
ABSTRACT: The horseradish peroxidase catalyzed aerobic oxidation of the auxin indole-3-acetic acid generates triplet indole-3-aldehyde in high yield. The excited species is quenched by oxygen with formation of singlet oxygen, which is responsible for the observed photon emission and can be trapped by suitable agents. tRNA dramatically enhances the emission as a result of energy transfer from triplet indole-3-aldehyde to a 4-thiouridine group in tRNA. Triplet indole-3-aldehyde also adds covalently to tRNA. The results provide a possible mechanism for the auxin–tRNA interaction in vivo.

The biochemical generation and functionality of electronically excited species—other than those involved in classical bioluminescence—are under investigation in our laboratories (Durán et al., 1977; Faria Oliveira et al., 1978; Cilento et al., 1978; Vidigal-Martinelli et al., 1979; Bechara et al., 1979; Rivas-Suarez et al., 1979; Augusto & Cilento, 1979; Durán et al., 1979; Makita & Durán, 1979; Vidigal et al., 1979; Cilento, 1980; Zinner et al., 1980; Durán & Cilento, 1980; Haun et al., 1980). A system which qualifies for investigation, and is being thoroughly investigated, is the peroxidase-catalyzed aerobic oxidation of IAA[1] to IAl (Vidigal et al., 1975, 1979; Durán et al., 1976; Zinner et al., 1976, 1980). The reaction proceeds, at least formally, through an intermediate dioxetanone (Cilento, 1975); since dioxetanone/dioxetane cleavage affords one of the carbonyl derivatives in an electronically excited state (Kopecky & Munford, 1969; McCapra, 1973; Turro et al., 1973; Bechara et al., 1976; Wilson, 1976; Adam, 1977; Horn et al., 1979), IAl might be generated excited:



IAA IAl

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[1] Abbreviations used: IAA, indole-3-acetic acid; IAl, indole-3-aldehyde; HRP, horseradish peroxidase; HRP-I, horseradish peroxidase compound I; HRP-II, horseradish peroxidase compound II; ISC, intersystem crossing; DABCO, 1,4-diazabicyclo[2.2.2]octane.

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IAA, which is probably the principal native auxin, may be active in concentrations as low as 10^{-8} M. Hence, there must be an amplification mechanism; one possibility is that in plant tissues IAA is activated and then attached to RNA (Kefford et al., 1963; Galston, 1974). When [2-^{14}C]IAA was exposed to the peroxidase system in the presence of RNA, decarboxylation occurred as expected, and, very interestingly, the labeled moiety was covalently linked to RNA (Bednar et al., 1976). Therefore, it is possible that activation of IAA consists of the generation of electronically excited IAl, which would then interact with RNA. If so, excited-state formation from IAA/HRP/O_2 may be biologically important [Cilento, 1965, 1973, 1980; see also White & Wei (1970)].

It is the purpose of the present study to substantiate the formation of electronically excited IAl by the IAA/HRP/O_2 system and to examine its interaction with tRNA.

Materials and Methods

Escherichia coli strain W tRNA, HRP (type VI), IAl, superoxide dismutase, catalase, and sorbic acid were obtained from Sigma Chemical Co.; IAA was from Merck, DABCO was from Aldrich Chemical Co., 2-methylpropanal was from Carlo Erba, and [2-^{14}C]IAA was from New England Nuclear (49 mCi/mmol). Anthracene-2-sulfonate, 9,10-diphenylanthracene-2-sulfonate, and 9,10-dibromoanthracene-2-sulfonate (sodium salts) were prepared by published methods (Faria Oliveira et al., 1978).

Oxygen consumption was measured with a Yellow Springs Instruments Model 53 oxygen monitor. Fluorescence spectra were obtained with a Perkin-Elmer MPF-4 fluorescence spectrometer.

Energy transfer studies with tRNA were carried out by the method of Beardsley & Cantor (1970), which takes advantage of the naturally occurring luminescent base. The chemiluminescence from the enzymatic system was measured in a Hamamatsu TV C-767 photocounter or a Beckman LS-100c liquid scintillation counter.

The labeling experiments were carried out as follows. The reaction mixture contained, in a final volume of 200 μL, 30 μM EDTA, 50 μM [2-^{14}C]IAA, and 500 μg/mL tRNA in 0.05 M acetate buffer, pH 3.8 or 5.6, or in 0.05 M phosphate buffer at pH 6.8; the reactions were initiated by the addition of 0.25 μM HRP and incubated at 25 °C for 15 min. The reaction was stopped by addition of an equal volume of phenol-saturated buffer and centrifuged, and an aliquot of the deproteinized aqueous fraction was added to a 5% trichloroacetic acid solution. The precipitate was filtered in a fiberglass filter apparatus. The filter was placed in scintillation vials containing a toluene scintillation cocktail. The amount of isotope was determined with a Beckman LS-250 liquid scintillation counter.

[^{14}C]IAl was obtained by preparative paper chromatography, Whatman 1, from the enzymatic reaction using 50 μM [2-^{14}C]IAA, 30 μM EDTA, and 0.25 μM HRP in 0.05 M acetate buffer, pH 3.8. 2-Propanol–NH_4OH–H_2O (13:1:1) was used as the eluant.

All the irradiations were carried out in the fluorescence spectrometer at 280 nm, in 1-cm fluorescence cells.

Results and Discussion

Reaction of HRP-Activated IAA with tRNA. Using C_2-labeled IAA, we have observed that the HRP-catalyzed aerobic oxidation in the presence of tRNA leads to radioisotope incorporation in the latter; from five experiments the percentage of incorporation was 4.5 ± 0.7. We have thus confirmed the

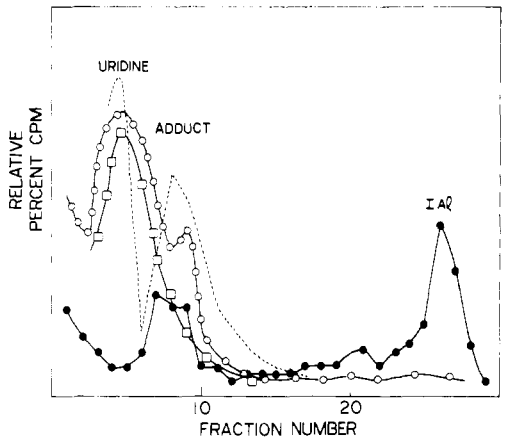


FIGURE 1: Adduct formation between optically or enzymically generated excited IAl and uridine. Two optical excitation experiments were run. In one of them, a mixture of 25 μM [^{14}C]IAl and 10^{-4} M uridine was excited at 280 nm (●); in the other, a mixture of 0.1 mM IAl and 1 nM [^{3}H]uridine was excited (○). For the enzyme experiment, 1 nM [^{3}H]uridine was added to the IAA (0.1 mM)/HRP (0.25 μM)/O_2 system (- - -). A control experiment was run with 1 nM [^{3}H]uridine (□).

results of Bednar et al. (1976) (they find 13.6% incorporation) despite the absence of added H_2O_2 in our system. If tRNA is added after the IAA oxidation has taken place, no radioisotope is incorporated unless the mixture is irradiated at 280 nm, 12 h of irradiation in the fluorometer with an open slit (20-nm bandwidth) inducing 18% incorporation. The latter result was also obtained by irradiating, at 280 nm, a mixture of IAl (labeled at C_1) and RNA. Since RNA absorbs much less at 280 nm than does IAl under our conditions, these results indicate that the species which adds to tRNA is electronically excited IAl, whether generated enzymically or optically. Less radioisotope was incorporated when the enzymic reaction was run at pH 5.6 than at pH 3.8; even less incorporation occurred at pH 6.8, in agreement with decreased IAl formation at higher pH values (Yamazaki et al., 1970).

It is possible that excited IAl adds to a uridine residue in tRNA. Thus, by adding [^{3}H]uridine to the IAA/HRP/O_2 system or irradiating a mixture of [^{3}H]uridine with IAl or a mixture of uridine with IAl labeled at C_1, one can detect the formation of a new product which appears to be the same in the three cases (Figure 1). No labeling was observed under the same conditions when guanine, cytosine, or adenosine was substituted for uridine. It may be that the addition of enzyme-generated excited IAl to RNA is responsible for the alterations observed in the CD spectrum of the latter (Vidigal et al., 1979). The alterations, although small, are highly reproducible and reminiscent of those observed as a result of the photobinding of psoralene to tRNA (Ou & Song, 1978).

The incorporation of radioisotope into tRNA, ascribed to reaction of excited IAl, implies that the quantum yield of chemiexcitation to IAl is no less than 5%.

Energy Transfer from the IAA/HRP/O_2 System to tRNA. When used at low concentrations (0.4–1.6 μM), tRNA quenches the emission from the IAA/HRP/O_2 system; however, at higher concentrations (80 μM), RNA greatly enhances the emission at both pH values investigated, 3.8 and 6.8. This indicates the existence of two types of excited species in the IAA/HRP/O_2 system, one of them being emissive and easily quenched at low tRNA concentration. As we shall see, it is likely that this species is $^1\Delta_g$ singlet oxygen, whereas the other one is triplet IAl.

The temporal behavior of the RNA-enhanced emission is presented in Figure 2. The enhancement only occurs when

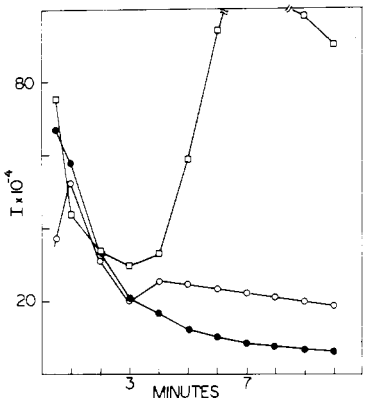


FIGURE 2: Temporal behavior of the 40 μM tRNA (upper curve) and of 39 μM 4-thiouridine 5'-phosphate (middle curve) sensitized emission from the IAA/HRP/O_2 system. The lower curve represents the emission in the absence of sensitizer.

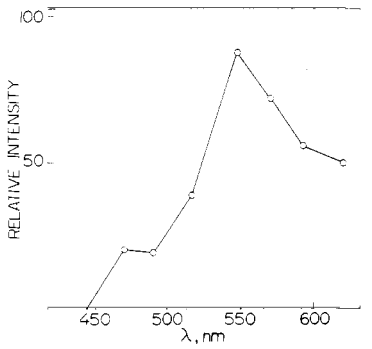


FIGURE 3: Spectral distribution of the 40 μM tRNA sensitized emission from the IAA (0.1 mM)/HRP (0.25 μM)/O_2 system. The direct emission is much weaker (not shown).

oxygen has been largely consumed, a fact which in turn suggests that either the donor or the emissive acceptor (or both) must be in the triplet state. As shown in Figure 2, 4-thiouridine 5'-phosphate at the concentration used (39 μM) had only a modest sensitizing effect, noticeable after extensive oxygen depletion.

The emission spectrum from the IAA/HRP/O_2/tRNA system (Figure 3) is dominated by a peak at ~550 nm, and there is also some indication of a small peak at ~475 nm. Energy transfer appears to populate the triplet state of a 4-thiouridine group in tRNA. Thus, at room temperature, 4-thiouridine phosphoresces maximally at 550 nm (Shalitin & Feitelson, 1973); in polar glasses, this maximum shifts to 520 nm upon lowering the temperature and a new peak starts developing at 475 nm (Figure 4); tRNA itself luminesces maximally at ~520 and ~440 nm when excited at 345 nm (Figure 5).

The species serving as the donor of excitation energy to thiouridine in tRNA must be enzyme-generated excited triplet IAl. Photophysical experiments support this inference. tRNA absorbs much less at 280 nm than IAl; if the latter is present, emission bands of tRNA are seen at 440 and 532 nm, in addition to the unchanged fluorescence of the aldehyde (Figure 5). Photoexcited IAl was capable of directly exciting the thiouridine base at both pH values investigated, 3.8 and 6.8.

The fact that the sensitized emission from tRNA is much stronger than with 4-thiouridine 5'-phosphate might indicate a more efficient transfer from enzyme-generated excited IAl to the macromolecule and/or that, in the latter, thiouridine is somewhat protected from deactivating oxygen collisions. At present, it is still premature to speculate as to the mechanism of energy transfer from enzyme-generated excited species; see,

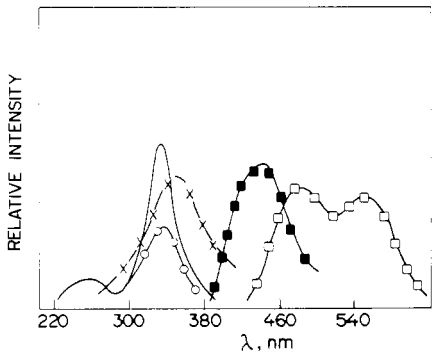


FIGURE 4: Optical data for 4-thiouridine 5'-phosphate. The curve (—) is the absorption spectrum whereas the curve (□) is the emission (phosphorescence) spectrum resulting from excitation at 335 nm at −130 °C. The curve (○) is the excitation spectrum for the emission at 550 nm. Also shown are the fluorescence (×) and phosphorescence (■) spectra of IAl; excitation was at 280 and 300 nm, respectively.

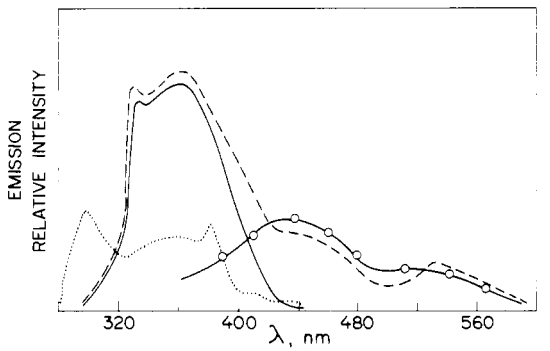
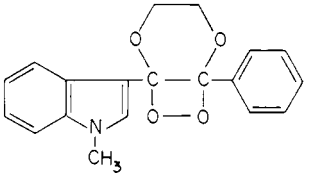


FIGURE 5: Energy transfer from excited IAl to tRNA. The curve (---) shows the emission spectra of a mixture of 0.1 mM IAl and 40 μM tRNA excited at 280 nm; at this wavelength, tRNA absorbs much less than does IAl. The curve (—) represents the emission spectrum of 0.1 mM IAl excited at 280 nm, whereas the curve (○) represents the emission spectra of 40 μM tRNA excited at 345 nm. The curve (···) is the excitation spectrum of tRNA for the emission at 440 nm.

however, Kasha (1979) and Cilento (1980).

The donor species, i.e., enzyme-generated excited IAl, might be generated in either a ^3n–π^* or a $^3\pi$–π^* state. The emission bands of tRNA are not seen when the IAA/HRP/O_2 is substituted by the 2-methylpropanal/HRP/O_2 system, which generates ^3n–π^* triplet acetone (Bechara et al., 1979), the only effect being quenching of the acetone phosphorescence. A further difference is the failure of the IAA/HRP/O_2 system to excite efficiently the 9,10-dibromoanthracene-2-sulfonate ion, in contrast to enzyme-generated ^3n–π^* acetaldehyde (Haun et al., 1980) and ^3n–π^* acetone (Faria Oliveira et al., 1978); as expected, the anthracene-2-sulfonate ion and its 9,10-diphenyl derivative were not excited at all.

Cleavage of the intermediate dioxetanone may give rise to



a π–π^* excited species; indeed, cleavage of the dioxetane leads to π–π^* excitation (Nakamura & Goto, 1979). There is, however, an important difference as cleavage of this dioxetane generates indole-type fluorescence (λ_{max} = 320 nm), whereas the system IAA/HRP/O_2 does not emit in the UV region. A possible explanation is a low yield of $^1\pi$–π^* IAl (compared to the excited singlet species formed by the Nakamura-Goto

Table I: Influence of Various Agents upon the Emission from the Peroxidase-Catalyzed Indole-3-acetic Acid Oxidation

compd		integrated emission at 1 min (counts) × 10^{-5}	remarks
control		5.3	
sorbic acid	2 mM	5.2	triplet quencher[a] E_T = kcal/mol
DABCO	20 mM	35.0	inhibition of $^1O_2$ formation and enhancement of $^1O_2$ emission[b]
	10 mM	15.2	
superoxide dismutase	1740 units	5.0	trap for $O_2^{-}\cdot$
catalase	125 units	6.1	trap for H_2O_2
benzoate	10 mM	6.2	trap for $HO\cdot$[c]
bicarbonate	10 mM	6.0	enhancement of the emission when the species is $CO_2^{-}\cdot$[d]

[a] Wagner, 1967; Wilson et al., 1976; Steinmetzer et al., 1973; Cornelisse & Havinga, 1975. [b] Deneke & Krinsky, 1976. [c] Beauchamp & Fridovich, 1970. [d] Hodgson & Fridovich, 1976; Nakano & Sugioka, 1977.

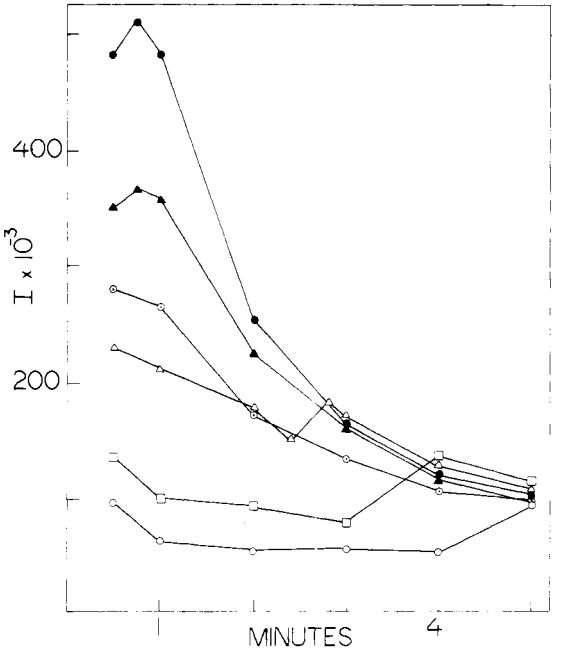


FIGURE 6: Quenching effect of increasing concentrations of histidine upon the emission from the IAA (0.1 mM)/HRP (0.25 μM)/O_2 system. From the top: no histidine; 1.25 mM; 2.5 mM; 5 mM; 10 mM; 20 mM.

dioxetane) coupled with very efficient ISC (Song & Kurtin, 1969).

Formation of Singlet Oxygen. Since triplet species are quenched by oxygen and since $^1O_2$ may be formed in such processes (Wu & Trozzolo, 1979), it was very important to look for $^1O_2$ formation. As a first approach, the effects of typical singlet oxygen quenchers, traps, or enhancers of its emission were assayed (Nilsson et al., 1972; Wasserman, 1970). Figure 6 shows the effect of histidine upon the emission in concentrations that do not influence the rate of O_2 uptake. A Stern–Volmer plot of the observed quenching effect is presented in Figure 7; the plot is linear, the slope K_{sv} (or $k_q\tau^0$) being 276 M^{-1}. If we assume that k_q is $\sim 10^8$ M^{-1} s^{-1} (the value observed for the reaction of $^1O_2$ with histidine) (Foote, 1976), τ^0 would be $\sim 2.8 \times 10^{-6}$ s; the latter value suggests that the excited species is indeed $^1O_2$, whose τ^0 in water is $\sim 2 \times 10^{-6}$ s. The reciprocal of the Stern–Volmer constant will correspond to β, the concentration of trap (histidine) necessary for sequestering 50% of $^1O_2$; the value agrees well with the reported value of 3×10^{-3} M (Foote, 1976). A similar investigation with guanosine yielded less accurate data, which, nonetheless, are consistent with the inference that the excited species is $^1O_2$.

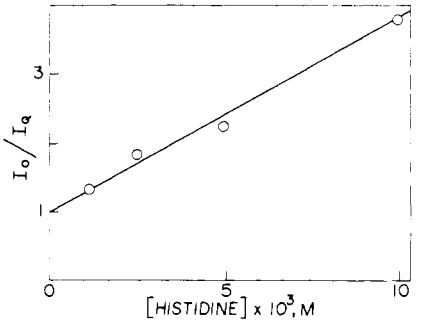


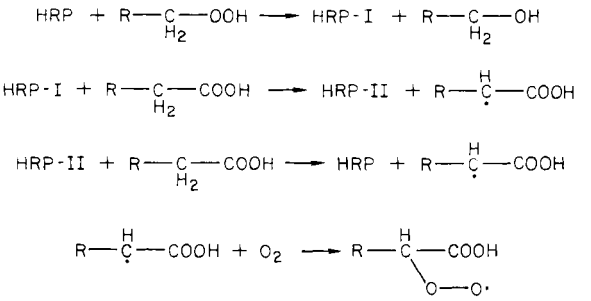
FIGURE 7: Stern–Volmer plot of the quenching of the emission from the IAA (0.1 mM)/HRP (0.25 μM)/O_2 system by histidine. Data were from Figure 6.

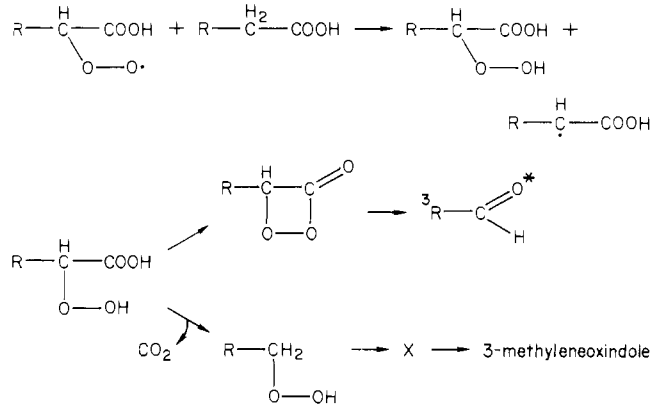
The emission spectrum from $^1O_2$ (bimol) is notoriously complex (Andersen et al., 1978). The emission spectrum from the IAA/HRP/O_2 system is also complex but contains, in addition to other peaks, a prominent peak at 630 nm and an indication of another one above 675 nm (the limit of the equipment), as expected from singlet oxygen emission. That $^1O_2$ is an important emitting species is further supported by the enhancement of the emission by DABCO (Deneke & Krinsky, 1976) and also by the failure to observe any emission above 360 nm in the fluorescence spectrum of the spent reaction mixture, that is, of the reacted IAA/HRP/O_2 system.

The effect of several agents upon the emission from the IAA/HRP/O_2 system is presented in Table I. The absence of a significant effect of superoxide dismutase is in accord with the fact that this enzyme does not affect the IAA oxidase activity of peroxidase (Nakajima & Yamazaki, 1979).

The product formed when uridine is exposed to the IAA/HRP/O_2 system is unlikely to be due to reaction of uridine with $^1O_2$ because the product behaves similarly to that formed by addition of IAl to uridine (Figure 1).

To account for the generation of excited triplet IAl in high yield, we tentatively amplify the mechanism of Nakajima & Yamazaki (1979) as shown in the scheme (in which R is the 3-indolyl group)



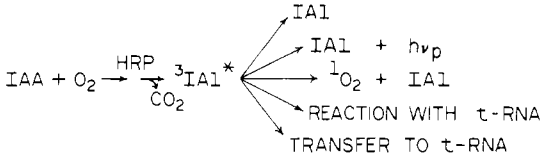


This scheme indicates that IAl and 3-methyleneoxindole come from the same intermediate (Ricard & Job, 1974); depending upon the experimental conditions, one pathway will be favored relative to the other.

At pH 3.8, O_2 consumption is faster than emission and IAl formation, the latter two phenomena correlating with each other (Vidigal et al., 1979). This indicates accumulation of the dioxetanone (and/or its hydroperoxide precursor) which in turn means that excited IAl should be formed outside the enzyme, i.e., in the bulk solution. This inference is consistent with the fact that enzyme-generated ^{3}IAl reacts with uridine and with tRNA.

Earlier, we reported that enzyme-generated excited species are able to excite a series of acceptors, including macromolecules such as DNA (Meneghini et al., 1978; Faljoni et al., 1978) and RNA (Vidigal et al., 1979). In the case of macromolecules, the occurrence of energy transfer was inferred. The observation of thiouridine phosphorescence when tRNA is added to the IAA/HRP/O_2 system conclusively shows that energy can indeed be transferred from enzyme-generated excited species to macromolecules with informational value.

The following scheme shows the competitive pathways open to ^{3}IAl in the presence of tRNA:



Both IAA (Kefford et al., 1963) and peroxidase (Wolter & Gordon, 1975) appear to be involved in the control or growth rate and differentiation of specific cell types. In addition to its role in protein synthesis, tRNA participates in other important processes such as the control of gene expression (Rich & Kim, 1978); in some systems, the control function appears to be associated with a particular modified nucleotide in the tRNA molecule. Indeed, one possibility for the regulators in plants is that they modify a particular variety of transfer RNA needed to initiate synthesis of a specific enzyme (Szabo et al., 1975; Armstrong, 1966). It is tempting to speculate that reaction of tRNA with enzyme-generated excited IAl may provide an example of this modification. Another modification of RNA might be provided by a reaction between the thiouridine group, excited by energy transfer (from triplet IAl), and a neighboring group: such a photoproduct indeed occurs in the irradiation of $tRNA_1^{Val}$ (Pochon et al., 1971).

In conclusion, our results provide a possible mechanism for the IAA–tRNA interaction in vivo.

Acknowledgments

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Reactions of Nitric Oxide with Cytochrome *c* Oxidase†

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ABSTRACT: The reactions of nitric oxide (NO) with both oxidized and reduced cytochrome *c* oxidase are reported. NMR and mass spectroscopy were utilized to determine the products of the reactions; EPR and optical spectroscopy were employed to determine the states of the enzyme produced in each of these reactions. It was found that the enzyme catalyzes the consecutive oxidation and reduction of NO. A different cycle was observed when NO was added to the reduced enzyme, to the oxidized enzyme, or to the oxidized enzyme in the presence of azide. It was possible to observe the state of the enzyme at several points in each of these three cycles by varying the concentration of NO. The reactions of NO all involved a one- or two-electron redox step and could be accounted for by the involvement of only cytochrome a_3 and Cu_{a_3}. On the basis of these results, a mechanism for the reduction of dioxygen by the enzyme is proposed in which cytochrome a_3 functions to anchor dioxygen and intermediates while remaining in the ferrous state, whereas Cu_{a_3} functions to accept electrons from cytochrome a/Cu_a and transfer them to dioxygen.

Nitric oxide (NO) has been utilized extensively as a spin probe of the structure of oxygen binding proteins (Yonetani et al., 1972). The great utility of NO is that in addition to closely resembling dioxygen it also has one unpaired electron, which can be used to transform an even-spin site into an odd-spin site observable by EPR.[1] For example, ferroheme proteins, which normally do not exhibit any EPR signals, are transformed into a nitrosylferroheme species which exhibits a diagnostic EPR signal upon the addition of NO (Yonetani et al., 1972). In several other enzymes including cytochrome *c* oxidase (Malmström, 1979), hemocyanin (Lontie & Vanquickenborne, 1974), and laccase (Fee, 1975), two closely associated metal atoms form the oxygen binding sites, and a strong antiferromagnetic exchange interaction between the two metal centers renders the oxidized state of these enzymes EPR silent. Again if NO were to bind to one of the two metal centers of such an antiferromagnetically coupled pair, it could be possible to disrupt the antiferromagnetic coupling to produce a state observable by EPR spectroscopy as we recently demonstrated in our study of the interaction of NO with the oxygen binding site of oxidized cytochrome *c* oxidase (Stevens et al., 1979a).

In this paper, we have examined more closely the interactions of NO with cytochrome *c* oxidase. It will be shown that several reactions of NO are catalyzed by this enzyme, to both oxidize and reduce NO, and that these reactions can be utilized to produce states of the enzyme in which both cytochrome a_3 and Cu_{a_3} exhibit EPR signals. The elucidation of these re-

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; PPD, *p*-phenylenediamine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; FT, Fourier transform; rf, radio frequency.

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