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CONCERNING THE FORMATION OF SINGLET O_2 DURING THE DECOMPOSITION OF H_2O_2 BY CATALASE

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SUMMARY

2,5-Diphenylfuran was used in a spectrophotometric assay for singlet O_2 to determine if this species were present during the reaction of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) with $^2H_2O_2$. Since 2,5-diphenylfuran is a better singlet O_2 trap in ^2H_2O than in H_2O, all measurements have been made in ^2H_2O. Under these conditions less than 0.5% of the total O_2 formed in the decomposition of $^2H_2O_2$ by catalase was released from the enzyme as singlet O_2.

INTRODUCTION

The decomposition of H_2O_2 by catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) has been proposed to occur either by two single-electron steps [1] or by a single hydride transfer [2, 3]. If there were no mechanism available for the enzyme to relax singlet O_2, the latter mechanism predicts that O_2 would be released from the enzyme in the excited-singlet state. Using N_2 as a trap for singlet O_2, Anbar [4] detected a species in the catalase reaction that exhibited the reactivity of singlet O_2. Jones and Suggett [3] have subsequently quoted this work as supporting evidence for a hydride-transfer mechanism. Unfortunately, the molecular dimensions of N_2 are similar to those of H_2O_2, making it probable that N_2 is capable of not only reacting with singlet O_2 free in solution but also reacting at the active site of the enzyme. Consequently these experiments do not distinguish between a "bound type" singlet O_2 and singlet O_2 free in solution. More recently, Kasha and Khan [5] reported chemiluminescence during H_2O_2 breakdown by catalase and have concluded that singlet O_2 is present. However, Kearns [6] has pointed out that the mechanism of chemiluminescence in singlet O_2 reactions is not completely understood and consequently is not a good criterion to use as evidence for the presence of singlet O_2. From a biological point of view it is unlikely that catalase would be fulfilling its protective role in the cell by breaking down H_2O_2 only to make the more reactive species, singlet O_2. In summary, conclusive evidence for singlet O_2 release from catalase during H_2O_2 decomposition has not been given. It is the purpose of this paper to estimate the fraction of O_2 released as singlet O_2 in the catalase reaction using the singlet O_2 trap 2,5-diphenyl-

furan. Since 2,5-diphenylfuran is a relatively large molecule it is sterically restricted from the active site of catalase. Thus, unlike N_2, 2,5-diphenylfuran will react with singlet O_2 free in solution and not with a reactive species at the active site of the enzyme.

MATERIALS AND METHODS

Catalase isolated from beef heart and twice recrystallized was purchased from Schwarz-Mann Corp. and used without further purification. 2,5-diphenylfuran was prepared by the method of Lutz and Rowlett [7] and purified by repeated recrystallizations from ethanol. (m.p. uncorrected = 89–89.5 °C, reported 89.5 °C [16].) The ultraviolet spectrum was identical to that reported by King et al. [8]. ^2H_2O (99.7% atom) and $^2H_2O_2$ was obtaned from Biorad. All other chemicals were reagent grade.

Singlet O_2 was generated in situ by shining a 100-W G.E. CDJ bulb at right angles to the analyzing light of a Cary 14 spectrophotometer through a 535-nm cutoff filter (Corning No. CS3-68) onto a cuvette containing methylene blue [9]. The formation of the hydroperoxide of 2,5-diphenylfuran was followed by the disappearance of 2,5-diphenylfuran absorbance at 324 nm [10]. p^2H values were measured on a Corning Model 12 pH meter making a suitable correction for ^2H_2O [11]. O_2 concentrations were measured with a Yellow Springs Instrument Company O_2 electrode and recorded on a Varian aerograph Model 10 recorder. The O_2 concentration in solution was taken to be 240 μM when air saturated [22].

In a system where singlet O_2 is an intermediate, the kinetics of the reaction of this species with 2,5-diphenylfuran (T) is described by the following scheme:

$$\xrightarrow{V_0} {}^1O_2 \xrightarrow{k_1} {}^3O_2 \qquad (1)$$
$$\Big\downarrow k_2[T]$$
$$TO_2$$

where V_0 is the rate of formation of singlet O_2 ($^1O_2$), k_1 is the first-order relaxation of singlet O_2 to triplet O_2 ($^3O_2$) by solvent, and $k_2[T]$ is the reaction of singlet O_2 with 2,5-diphenylfuran to yield the hydroperoxide, TO_2. In this scheme the steady-state concentration of singlet O_2 is

$$[^1O_2]_{ss} = \frac{V_0}{k_1 + k_2[T]} \qquad (2)$$

The quantity β is defined as the ratio of k_1 to k_2. If the concentration of T were equal to β, then 50% of the singlet O_2 formed through V_0 in Eqn 1 would be trapped as TO_2. Under conditions when β is much greater than the concentration of T Eqn 2 reduces to

$$[^1O_2]_{ss} = \frac{V_0}{k_1} \qquad (3)$$

If V_0 is constant, the change of concentration of T with time will be a first-order process described by

$$\frac{dT}{dt} = -k_2[^1O_2]_{ss}[T] \qquad (4)$$

Substituting Eqn 3 into Eqn 4 one obtains

$$\frac{dT}{dt} = -k_{obs}[T] \tag{5}$$

where

$$k_{obs} = \frac{V_0}{\beta} \tag{6}$$

Since the β value of 2,5-diphenylfuran is known to be $4.6 \cdot 10^{-4}$ M in H_2O [13], the rate of singlet O_2 formation in H_2O can be calculated from the k_{obs} for 2,5-diphenylfuran disappearance and Eqn 6. Conversely if V_0 is independent of solvent the β value of 2,5-diphenylfuran can be obtained in a different solvent system by the ratio of k_{obs} in the new solvent with that in H_2O. This is formulated in Eqn 7

$$\beta_x = \frac{\beta\, k_{obs}}{k^x_{obs}} \tag{7}$$

where β and k_{obs} are for H_2O and k^x_{obs} is the measured rate of 2,5-diphenylfuran disappearance in the unknown solvent. For Eqn 7 to be valid β must be greater than the 2,5-diphenylfuran concentration.

RESULTS

Eqn 5 demonstrates that the smaller the β value the more sensitive 2,5-diphenylfuran will be as a singlet O_2 trap. Recently, Merkel and Kearns [9] have shown that the lifetime of singlet O_2 is 10-fold longer in ^2H_2O than it is in H_2O. If there were little effect of ^2H_2O on k_2, the β value of 2,5-diphenylfuran would be significantly reduced in ^2H_2O. Since catalase is known to be active in ^2H_2O, it was of interest to measure the β value of 2,5-diphenylfuran in ^2H_2O.

Table I presents β values for 2,5-diphenylfuran determined in ^2H_2O with light and methylene blue as the source of singlet O_2. The following assumptions have been made to calculate these values. First it is assumed that V_0 is constant and does not change with solvent conditions. This assumption has been used and justified by previous workers [9, 13]. In addition, it is assumed that V_0 will not change from the decrease of O_2 due to 2,5-diphenylfuran oxidation during each experiment. This is a reasonable assumption since the 2,5-diphenylfuran concentration is less than 2% of the total O_2 concentration. Finally it is assumed that β is greater than the concentration of 2,5-diphenylfuran. The fourth column of Table I is the ratio between the calculated β values and the initial 2,5-diphenylfuran concentration. This ratio is always greater than twenty and proves that the original assumption was valid.

The results of Table I show that the β value for 2,5-diphenylfuran in ^2H_2O is 8.7 times less than the β value in H_2O. Also 0.01 M phosphate buffer increases the β value slightly while catalase and $^2H_2O_2$ have little effect on β. With the values of Table I and the k_{obs} for 2,5-diphenylfuran disappearance it is now possible to calculate V_0 from Eqn 6 for a system forming singlet O_2. This is now applied to the catalase reaction.

Since the β value of 2,5-diphenylfuran in ^2H_2O is less than it is in H_2O, the

TABLE I

β VALUES FOR 2,5-DIPHENYLFURAN UNDER VARIOUS REACTION CONDITIONS

In our hands a saturated solution of 2,5-diphenylfuran in ^2H_2O was 0.55 μM. The concentration of 2,5-diphenylfuran used in these experiments was 2.6 μM. To obtain this concentration, a concentrated solution of 2,5-diphenylfuran was made in acetone. A small aliquot of the acetone solution was added to the solvent to give a 2.6-μM 2,5-diphenylfuran solution. 2,5-diphenylfuran precipitation was slow as judged by disappearance of 324-nm absorbance. The final acetone concentration was 13 mM. k_{obs} is first-order rate constant for 2,5-diphenylfuran disappearance using methylene blue (A_{662} = 0.22) and light as a source of singlet O_2. Solutions were air saturated at 23 °C. Average of three experiments. 2,5-diphenylfuran concentrations calculated using $\varepsilon_{324} = 29\,000\ M^{-1}\cdot cm^{-1}$ from ref. 8.

Solvent	k_{obs} (s^{-1})	β (M)	β/2,5-diphenylfuran
H_2O	$2.48\cdot10^{-3}$	$4.58\cdot10^{-4}$*	172
^2H_2O	$2.15\cdot10^{-2}$	$5.27\cdot10^{-5}$**	19
^2H_2O + 0.01 M potassium phosphate (pH 7.1)	$1.33\cdot10^{-2}$	$8.35\cdot10^{-5}$**	30
^2H_2O + 0.01 M potassium phosphate (960 μM ^2H_2O, pH 7.1)	$1.20\cdot10^{-2}$	$9.45\cdot10^{-5}$**	34
^2H_2O + 0.01 M potassium phosphate (1 μl/ml catalase, pH 7.1)	$1.50\cdot10^{-2}$	$7.57\cdot10^{-5}$**	27

* β value taken from reference 13.
** β values calculated by Eqn 7 in text.

conditions chosen for trapping singlet O_2 in the catalase reaction are 0.01 M phosphate buffer in ^2H_2O at a pH of 7.1. The results of adding catalase to $^2H_2O_2$ in the presence of 2,5-diphenylfuran are shown in Fig. 1. During the course of O_2 evolution in the catalase decomposition of $^2H_2O_2$ there is essentially no disappearance of absorbance at 324 nm. Since the β value of 2,5-diphenylfuran in this solvent is known from Table I, a predicted rate of 2,5-diphenylfuran disppearance can be calculated. If one assumes that V_0 or rate of singlet O_2 production is equal to the rate of O_2 production measured on the O_2 electrode, the predicted decay of 2,5-diphenylfuran is given by the dashed line in Fig. 1 where the first-order rate constant describing 2,5-diphenylfuran disappearance is V_0/β. Also shown in Fig. 1 is the decrease of 2,5-diphenylfuran if only 5 % of the O_2 released from the enzyme were singlet O_2. If less than 0.5 % of the total O_2 formed from $^2H_2O_2$ were singlet O_2, we would observe no decrease in 2,5-diphenylfuran absorbance. To ensure that 2,5-diphenylfuran was acting as a singlet O_2 trap in the above experiment, methylene blue was added at the end of the reaction and light was directed onto the cuvette. The rate of 2,5-diphenylfuran disappearance was that predicted if no catalase or $^2H_2O_2$ had been added to the reaction mixture. Since Table I shows that neither catalase nor $^2H_2O_2$ effect the β value of 2,5-diphenylfuran in ^2H_2O, we conclude from our results with catalase that less than 0.5 % of the total O_2 released by catalase during its reaction with $^2H_2O_2$ is singlet O_2.

CONCLUSION

Table I and Fig. 1 demonstrate that only an extremely small fraction, if any, of the total O_2 released in the catalase reaction is free singlet O_2. Even though no

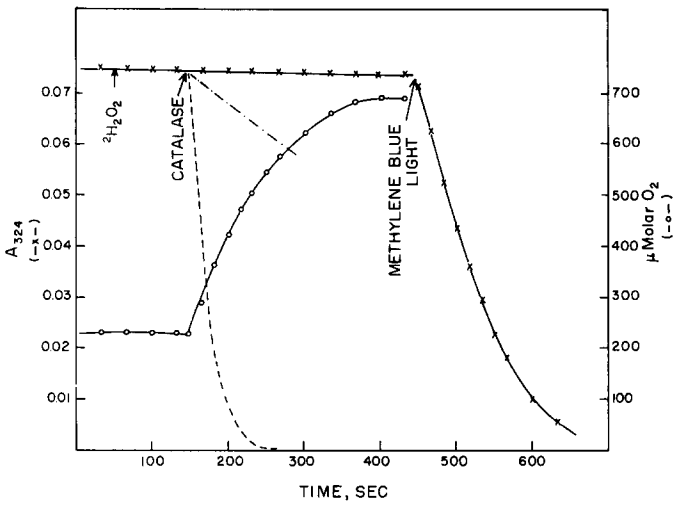

Fig. 1. Demonstration that no singlet O_2 is experimentally detectable in the decomposition of $^2H_2O_2$ by catalase. Two experimental traces are shown. That labelled with the crosses (-×-) refers to the absorbance of 2,5-diphenylfuran at 324 nm. The trace labelled with the open circles (-○-) refers to the production of O_2 measured on the O_2 electrode. Initially 2.6 μM 2,5-diphenylfuran was added to 0.01 M potassium phosphate buffer in ^2H_2O at a pH 7.1. At 50 s 0.940 mM $^2H_2O_2$ was added followed by 0.5 μg/ml catalase at 150 s. Finally, at 450 methylene blue (A_{662} = 0.32) was added and light directed onto the cuvette to generate singlet O_2. The broken line is the predicted curve if 5% of the total O_2 were released as singlet O_2 while the dashed line is calculated if all the O_2 were released as singlet O_2. Values used to calculate these lines are: β, 84 μM (average of the three β values in ^2H_2O and 0.01 M potassium phosphate given in Table I) and V_0, = 3.4 μM O_2/s if all O_2 released is singlet O_2 or 0.17 μM O_2/s if 5% of the O_2 is released as singlet O_2. Eqn 6 was used to calculate k_{obs}.

singlet O_2 is detected during the decomposition of H_2O_2 by catalase, it cannot be concluded that the mechanism of catalase action is two one electron transfers. There are two reasons for this, Merkel and Kearns [9] have shown that the lifetime of singlet O_2 is extremely dependent upon solvent. In particular the lifetime of singlet O_2 is directly correlated with the extinction coefficient of the solvent at 7880 cm^{-1}. If a hydride-transfer mechanism occurred in the catalase reaction, the enzyme might quench the singlet O_2 formed by placing a group with absorbance at 7880 cm^{-1} near the active site. For example, an amine similar to trimethylamine which quenches singlet O_2 with a bimolecular rate constant of $1.2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ [14] may be near the active site of the enzyme. Since the V of catalase is $6.6 \cdot 10^7$ s^{-1} [15] the effective concentration of amine in this case would have to be over 100 M for effective quenching of singlet O_2 to occur. The second effect that could account for the production of triplet O_2 in the catalase reaction even though a hydride-transfer mechanism is operative may be due to the heme center itself. If a two-electron transfer mechanism were operative, the resultant O_2–iron complex could dissociate into triplet O_2 and quartet iron. Because of large spin-orbital coupling [16] the quartet iron may relax rapidly to the sextet state for another reaction cycle.

In summary, we have found that the only form of O_2 released free in solution from catalase during H_2O_2 breakdown is triplet O_2. Because of several possible relaxation pathways for singlet O_2 this result does not distinguish between a two-electron or two single-electron transfer mechanisms.

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