Hydroxyl Free Radical Formation from Hydrogen Peroxide by Ferrous Iron–Nucleotide Complexes†

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ABSTRACT: Oxygen free radicals are implicated in many biological processes and as etiological agents of certain pathological conditions. Hydroxyl free radicals react readily and indiscriminately, causing damage. Ferrous iron catalyzes hydroxyl free radical formation from hydrogen peroxide. The ability of Fe(II) to carry out this reaction depends upon the particular ligands of the metal ion. The present study examines the ability of the adenosine nucleotides to aid in the apparent ligation of added Fe(II) to catalyze H_2O_2-dependent formation of hydroxyl free radicals detected by the spin-trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO). The results demonstrate that Fe(II)-catalyzed OH formation from H_2O_2 in the presence of ADP, and more so in the presence of ATP, was on the order of 20–50-fold higher than in the absence of the nucleotides. AMP was without effect in this system. Ferric ion was considerably less effective, on the order of one-fifth to one-tenth as effective as Fe(II), in the nucleotide system. The dissolved gases had a drastic effect, with nearly a 10-fold decrease in the amount of OH produced from H_2O_2 in the ADP–Fe(II) system when only O_2 was present vs. in the presence of only N_2. The hydroxyl free radical scavengers thiourea, ethanol, and ethylene glycol either completely abolished OH formation or decreased the amount considerably as well as yielded spin-trapped scavenger spin adducts. the ADP–Fe(II)–H_2O_2 system yielded 20 μM hydroxyl spin adduct when H_2O_2 and 2 mM ADP were present and 100 μM Fe(II) was added as the limiting component. Ascorbate reduced the ferric–nucleotide complex to the ferrous complex which then was catalytically effective in forming OH from exogenously added H_2O_2 in high yield. The results of this study may have some important implications for the understanding of oxygen free radical damage to biological systems.

Oxygen free radicals have been demonstrated in many biological processes and implicated as etiological agents in several pathological conditions (Fridovich, 1978). Superoxide and hydroxyl free radical are two oxygen free radical species of consequence in biological systems. Normally, the enzymes catalase, superoxide dismutase, and glutathione peroxidase maintain superoxide and hydrogen peroxide at low levels in biological systems, but under certain circumstances, higher levels of these compounds may be attained, thus leading to oxidative damage.

Hydroxyl free radicals are extremely reactive either abstracting hydrogen from or adding hydroxyl to biological molecules in diffusion-controlled reactions. Hydroxyl free radicals can be produced from hydrogen peroxide in a Fenton-type reaction, classically written as such:

$$H_2O_2 + Fe(II) \rightarrow OH + OH^- + Fe(III)$$

Until quite recently, it has been almost impossible to study OH formation because of the difficulty in observing this free radical per se. But the spin-trapping technique (Janzen, 1971) has provided a great aid in this regard, especially in the use of one spin trap, DMPO,[1] which reacts with OH rapidly, 3.4×10^9 M^{-1} s^{-1} (Finkelstein et al., 1980), to yield a paramagnetic spin adduct which can be quantified.

Very little ferrous iron is present in vivo in the free ionic form, but it may be present either ligated to various biological compounds or bound to biological surfaces. The nature of the Fe(II) ligand is very important in determining whether OH will be formed from H_2O_2. For instance, I found that Fe(II) addition to a solution containing EDTA (ethylenediaminetetraacetate) yielded only a small amount of OH from H_2O_2, but Fe(II) addition to a solution containing DETAPAC (diethylenetriaminepentaacetate) yielded large amounts of OH from H_2O_2 (Floyd, 1982) and even more so when DNA was present (Floyd, 1981). Thus, it was considered important to determine if ferrous ions would complex with various biologically important molecules to form an association which would be able to catalyze OH formation from H_2O_2. Of the many biologically important molecules tested, many were without effect, but in the course of this investigation, we made a surprising observation; the di- and triphosphate esters of adenosine were quite effective in complexing with Fe(II) such as to catalyze OH formation from H_2O_2. This has not been observed previously and may be of importance in understanding the conditions necessary for oxygen free radical damage in biological systems.

Experimental Procedures

The nucleotides used were purchased as the sodium salt of the highest grade possible from Boehringer Mannheim GmbH and Sigma Chemical Co. The experiments reported have been repeated several times for each nucleotide during a period of more than 1 year. Because of instability after lengthy storage, nucleotides were utilized within a week to a few months after receipt. Inconsistent results with different aged lots necessitated this procedure. In all cases, the nucleotides were freshly weighed out, diluted with water up to 20 mM, and utilized within 24 h of preparation. Less concentrated solutions of nucleotides were prepared immediately before use (1–2 min) by dilution from the 20 mM stock. In the experiments reported, where comparisons in the amounts of hydroxyl free radical formed for each nucleotide is necessary, a standard

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[1] Abbreviations: AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; DETAPAC, diethylenetriaminepentaacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPR, electron paramagnetic resonance.

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ADP solution was used for normalization; that is, the standard ADP solution was prepared by water addition to ADP, sodium salt from equine muscle grade X obtained from Sigma, to yield 20 mM. A batch of this freshly obtained ADP was then divided into 200-μL aliquots frozen rapidly on dry ice and stored at −20 °C. Each experiment conducted with a different nucleotide was then compared with the results obtained with a thawed aliquot of the standard ADP batch. The results obtained over the course of more than 40 days utilizing one batch of ADP was remarkably constant, never varying over 10% on a day-to-day basis. Experiments conducted with nucleotides from one company conducted as much as 6 months previously were, in most cases, superimposable with similar experiments conducted with the same nucleotide obtained from another company. This is pointed out in the results when applicable.

The spin-trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) was synthesized as described previously (Floyd & Soong, 1977). After distillation, it was stored in small lots as the amorphous white solid under N_2 atmosphere at −20 °C until used. For stock solutions, the white solid was melted at 37 °C, diluted 10 to 1 with water, and filtered through water-washed activated charcoal utilizing 0.5 g of charcoal for 5 mL of a DMPO stock solution (Floyd & Wiseman, 1979). The DMPO present was determined by optical absorbance (7.22 mM^{-1} cm^{-1} at λ 226 nm). The DMPO stock solutions were sparged with N_2, protected from light, and stored at 4 °C (Floyd & Wiseman, 1979).

Ferrous ion addition to solutions was as ferrous ammonium sulfate in initial experiments, and, then in later experiments, as ferrous chloride. Both salts yielded qualitatively the same results, but the chloride salt was chosen to avoid possible complications with ammonium ions as a possible ligand to iron. Stock solutions of 0.1 M ferrous chloride were prepared by addition of 0.0012 N HCl to the salt followed by sparging with nitrogen gas. The stock solutions yielded identical results for 3–4 days. Working solutions of 1 mM $FeCl_2$ in 0.0012 N HCl were prepared immediately before use by diluting the stock solutions. Working solutions of H_2O_2 were prepared by dilution of the 30% stock. In the experiments reported which utilized ascorbate, a stock solution of 0.1 M ascorbic acid in H_2O prepared fresh was diluted immediately before use to a concentration of 1 mM.

A typical experiment would be carried out with careful attention to detail as follows: to 50 μL of buffer (100 mM NaCl–25 mM $NaHCO_3$, pH 6.7) was added 20 μL of 780 mM DMPO, and then 10 μL of nucleotide solution; after mixing, 10 μL of 1 mM $FeCl_2$ was added, immediately mixed, and then placed in a 37 °C shaking water bath for 30 s at which time 10 μL of 0.3% H_2O_2 was added and the contents were mixed rapidly and then incubated for 30 s at 37 °C. The sample was then transferred to a heat-sealed transfer pipet and an EPR spectrum started immediately. The time elapsed before the EPR analysis was started was about 1 min after H_2O_2 addition. The EPR spectra were obtained on a Varian E-109 X-band spectrometer. The instrumental parameters typically were as follows: magnetic field modulation 100 KHz with an amplitude of 2 G, 9.14-GHz microwave frequency, scan rate 100 G/8 min, time constant 0.3 s, temperature 25 °C, and incident microwave power 25 mW. It should be noted that experiments conducted with other buffers such as potassium phosphate, trihydroxyaminomethane, and Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] at pH values from 6.7 to 7.8 yielded qualitatively similar results as reported here.

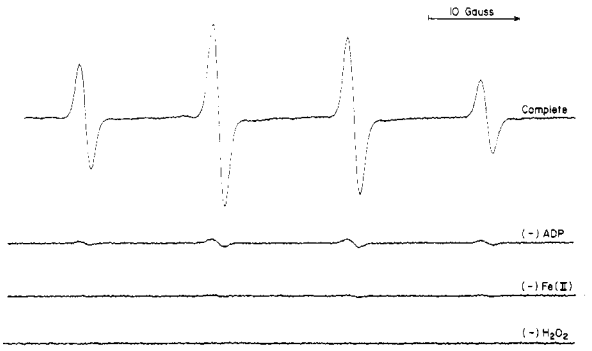

FIGURE 1: Electron paramagnetic resonance spectra of the hydroxyl free radical–DMPO spin adduct obtained in the Fe(II)–ADP–H_2O_2 system carried out as described under Experimental Procedures. The top spectrum was obtained in the complete system whereas the second, third, and fourth spectra down were obtained in the absence of added ADP, Fe(II), and H_2O_2, respectively. The instrumental parameters are listed under Experimental procedures.

Certain experiments were designed to ensure that the gases dissolved in the liquid phase were either only nitrogen or oxygen, respectively. They were conducted as described in the paragraph above only buffer, DMPO, and nucleotide solutions were added to a sealed transfer pipet and a stream of either nitrogen or oxygen was delivered to the tip of the pipet by using a finely pulled glass capillary. The stream of gas ensured that all of the liquid remained above the tip portion of the transfer pipet. This allowed subsequent additions to the gas-sparged solution with the assurance that the additions would be rapidly mixed. The buffer–DMPO–nucleotide solution in the transfer pipet was bubbled with gas for 10–15 min before N_2-sparged Fe(II) solution, and then N_2-sparged H_2O_2 was added according to the protocol described in the previous paragraph.

EPR observations at 77 K were conducted by using a protocol very similar to that of Burger et al. (1981b) in their investigation of iron ligation to bleomycin. That is, ethylene glycol was added after the hydroxyl free radical production had ceased (30 s), to a final amount of 50% (v/v), and the sample was frozen in liquid nitrogen immediately. The amount of free radical present was calculated by hand integration of the first derivative spectrum utilizing the Reimann sum method as described before (Floyd et al., 1978a). The standard utilized was the stable nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy. Instrumental parameters were maintained constant in the comparison of the standard with the hydroxyl radical spin adduct except the modulation amplitude was varied such that the value used in reference to the inherent line width was constant between the two.

Results

Figure 1 illustrates quite clearly the effect that ADP has on the amount of OH spin trapped when Fe(II) is added to the nucleotide-containing solution followed by H_2O_2 addition as described under Experimental Procedures. That is, the characteristic 1:2:2:1 EPR spectrum of the DMPO spin-trapped hydroxyl free radical with $A_N = A_\beta^H = 14.92$ G (Floyd et al., 1978b; Harbour et al., 1974) was quite evident when ADP, Fe(II), and H_2O_2 were present but was substantially reduced in amount when ADP was not present. The figure also illustrates that very little trapped OH was obtained unless Fe(II) as well as H_2O_2 was present. Figure 2 shows the effect of DMPO concentration on the amount of OH spin trapped in the 2 mM ADP–100 μM Fe(II) system described above. The amount of OH spin trapped increased in a biphasic manner with respect to DMPO, first rapidly and then more slowly at higher concentrations than 20 mM. We routinely

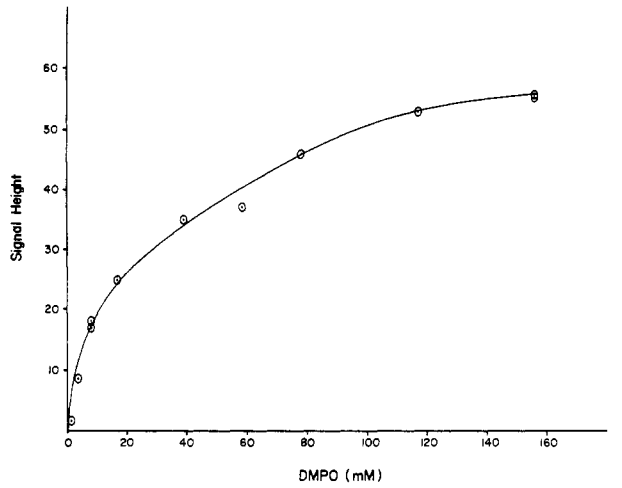

FIGURE 2: Effect of DMPO concentration on the amount of OH spin trapped in the Fe(II)–ADP–H_2O_2 system. The experiments were conducted as in Figure 1 except as proportionally less 780 μM DMPO was utilized, the volume was maintained constant by increased amount of H_2O.

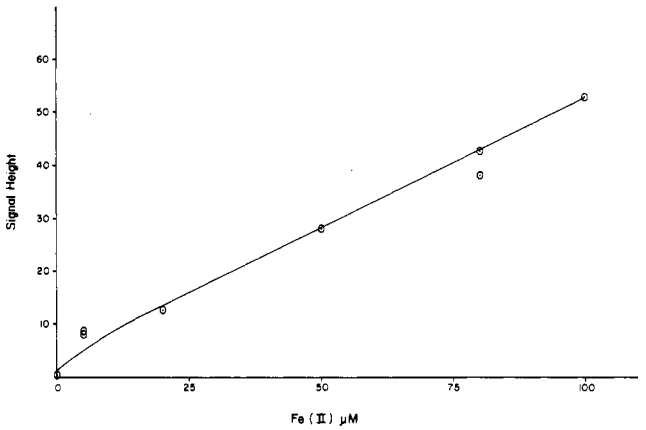

FIGURE 3: Effect of Fe(II) concentration on the amount of OH spin trapped in the Fe(II)–ADP–H_2O_2 system. The experiments were conducted as in Figure 1 except as proportionally less 1 mM FeCl was utilized, the volume was maintained constant by increased amount of 0.0012 N HCl.

used the highest concentration (156 mM) shown on this graph in our experiments. Other experiments conducted with higher DMPO concentrations illustrated that this concentration yielded the highest amount of trapped OH and at concentrations twice this value (312 mM), there was actually a slight decrease in the amount of OH spin trapped.

The effect of H_2O_2 concentration on the amount of OH spin trapped was examined. It was found that final values of H_2O_2 from 0.0003 to 0.03% yielded essentially the same amount of OH spin trapped (data not shown). This is as expected since H_2O_2 was more than 10-fold excess over the amount of Fe(II) even at the lowest level of H_2O_2 tested. Ferrous iron was added as the limiting component in the ADP–Fe(II) system, and this is reinforced by the results shown in Figure 3. The amount of OH spin trapped increased in a nearly linear fashion with the amount of Fe(II) added.

To further characterize the system, we have investigated the effect of dissolved gases, oxygen or nitrogen, on the amount of OH spin trapped in the ADP–Fe(II)–H_2O_2 system. The results obtained were as such. The amount of OH spin trapped in a nitrogen-sparged system was 15 times that trapped in the oxygen-sparged system. There was a 12.5% decrease in the amount spin trapped when air was present as compared to nitrogen. That is about 20 μM OH was spin trapped in an

Table I: Effect of Hydroxyl Free Radical Scavengers on the Amount Spin Trapped in the ADP-Fe(II)-H_2O_2[a] System

expt	scavenger	final concn	OH radical (μM)	scavenger radical
21/7	none		20.5	
7/7A	none		33[b]	
7/7C	ethanol	10%	7.5	5.5
7/7B	ethylene glycol	10%	10.5	8.5[c]
7/7D	thiourea	10 mM	0	

[a] The experiments were carried out based on the protocol described under Experimental Procedures. The final concentrations of ADP and H_2O_2 were 2 mM and 0.03%, respectively. [b] The values given for all experiments except 21/7 are all relative signal heights but comparable to each other. [c] The coupling constants of the ethylene glycol trapped radical is very similar to that of ethanol radical.

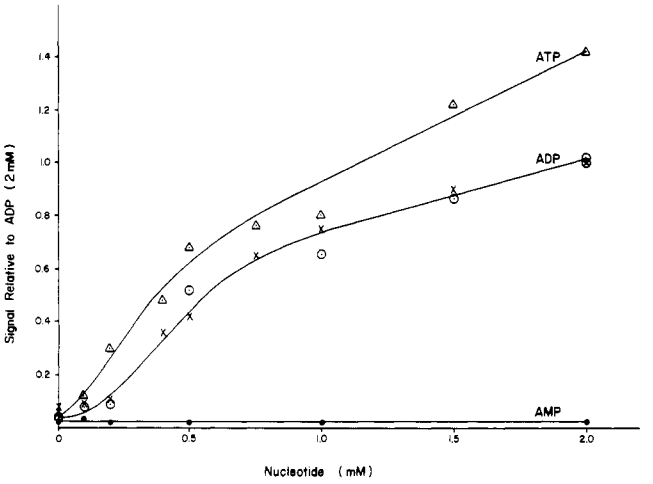

FIGURE 4: Effect of varying the adenosine nucleotide on the amount of OH spin trapped. The amount of nucleotide was varied by dilution of the 20 mM stock about 1–2 min before the reaction was started. The actual spin-trapping experiments were conducted as in Figure 1. The ADP was the standard ADP as described in the text obtained from Sigma Chemical Co., and the data collected from this ADP are shown in the open circles, whereas the ADP represented by (×) was obtained from Boehringer Mannheim GmbH. The ATP was the disodium salt from equine obtained from Sigma Chemical Co. The AMP was the sodium salt purchased from Sigma Chemical Co.

air-exposed system (see below) where 25 and 1.6 μM were spin trapped in the nitrogen-sparged and oxygen-sparged systems, respectively. Hence, it is apparent that the oxygen content of the media drastically affects the ability of added Fe(II) to catalyze OH formation from H_2O_2 in the ADP ligation system.

Utilizing TMPN as the standard, we have calculated that about 20.5 μM OH was spin trapped in a system having 2 mM ADP, 0.03% H_2O_2, and 100 μM Fe(II) added. This is shown in Table I. Table I also demonstrates that if ethanol or ethylene glycol was present in the system, a substantial amount of scavenger free radical was spin trapped with a concomitant reduction in the amount of OH spin trapped. This is as expected. Several other OH scavengers have been investigated including thiourea and mannitol. Thiourea at 10 mM completely eliminated any OH spin adduct formation. We do not understand why 10 mM thiourea was this potent as compared to 156 mM DMPO. Mannitol and other scavengers reduce the amount of OH spin trapped (data not shown).

Figure 4 demonstrates the effect of ADP as well as AMP and ATP concentration on the amount of OH spin trapped when Fe(II) and H_2O_2 levels were held constant. The curve obtained was with ADP purchased from two different com-

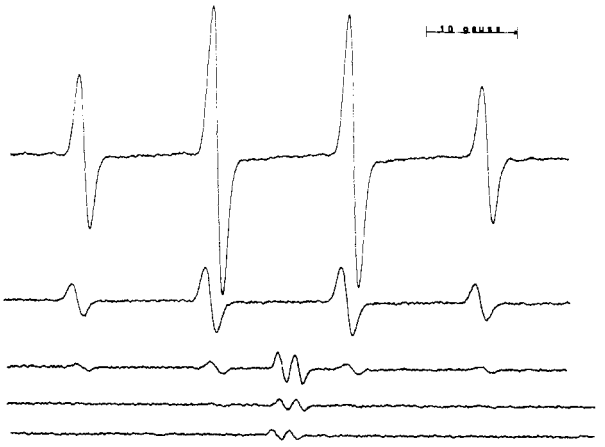

FIGURE 5: Electron spin resonance spectra demonstrating the effect of ascorbate addition to the Fe(III)–ADP complex upon the amount of OH spin trapped from H_2O_2. The experiments were conducted exactly as under Experimental Procedures with the exception that $FeCl_3$ in 0.0012 N HCl was used in place of $FeCl_2$ and that ascorbate (100 μM final concentration) was added and then the sample incubated at 37 °C for 30 s prior to the addition of H_2O_2 (top spectrum). In the second spectrum H_2O replaced ascorbate. In the third spectrum ADP and ascorbate were present, but no $FeCl_3$ was added. In the fourth spectrum ADP was not present, but $FeCl_3$ and ascorbate were added. In the bottom spectrum ADP–Fe(III) and ascorbate were added, but no H_2O_2 was added.

panies; one was made up fresh immediately after arrival, and the ADP solution used as the standard (open circles) was prepared and frozen 47 days before use as described previously. The sigmoidal type curve has been obtained repeatedly for ADP with several buffer systems over a pH range of 6.7–8.0.

Figure 4 also demonstrates some other very interesting results; namely, AMP was without effect in facilitating Fe(II)-catalyzed OH formation from H_2O_2 whereas ATP was more effective than ADP at all levels. This result is quite dramatic and has been verified several times to ensure its accuracy. In addition, we have found that the guanosine, thymidine, and cytosine nucleotides give qualitatively similar results as AMP, ADP, and ATP. That is, the di- and more so the triphosphate esters of these nucleotides were effective in ligation with Fe(II) in catalyzing OH formation from H_2O_2, but the monophosphate nucleotides were not. These results will be reported elsewhere. With reference to AMP, we have noted that aged batches of this nucleotide in some cases yielded increased OH production above the buffer control at the highest level, but subsequent investigation illustrated that adenosine itself slightly enhanced OH formation at the highest levels. Therefore, our interpretation of the aforementioned aberrant results with aged batches of AMP would be due to hydrolysis of AMP-yielding adenosine.

The oxidation state of the iron ions added had a large effect on the amount of OH spin trapped from H_2O_2 in the ADP system. Iron added in the ferric form was only one-tenth to one-fifth as effective as the ferrous ion. That is, the relative signal height with 100 μM $FeCl_2$, $FeCl_3$, and $FeNH_4(SO_4)_2$ was 41.0, 9.0, and 4.5, respectively.

The results shown in Figure 5 illustrate that ascorbate added to the ferric–ADP system reduced the ferric complex to the ferrous complex which was then capable of reacting with H_2O_2 to yield OH in high yield (top spectrum). The amount of ascorbate added was equivalent to the Fe(III) concentration, i.e., 100 μM. The second spectrum down shows the yield of OH obtained when H_2O_2 was added to the Fe(III)–ADP system in the absence of ascorbate. Ascorbate in the presence of ADP in the absence of added Fe(III) yielded very little OH from H_2O_2 (third spectrum down); Fe(III) in the absence of ADP to which ascorbate was added yielded very little OH from H_2O_2 (fourth spectrum down). As a control the system ADP–Fe(III) plus ascorbate but in the absence of added H_2O_2 yielded very little OH (bottom spectrum). The doublet from the ascorbate is readily apparent in the three bottom spectra. This set of experiments illustrates an important point, namely, that the Fe(III)–ADP complex can be reduced to the Fe(II)–ADP complex with ascorbate which then reacts readily with H_2O_2 to produce the OH in high yield.

Discussion

The work reported here clearly demonstrates for the first time that Fe(II) ligated with ADP as well as ATP, but not AMP, is quite effective in the catalysis of H_2O_2 to yield hydroxyl free radicals. This may be of importance intracellularly in the formation of damaging oxygen free radicals where conditions exist favorably for this occurrence. Normally, it is considered that ATP exists in the cell as the Mg complex (Merritt et al., 1978), yet there is evidence that Fe–nucleotide complexes do exist also in vivo (Hochstein, 1981). The demonstrations by Konopka (1978) and Konopka & Romslo (1980) that iron is mobilized from transferrin to mitochondria by phosphate compounds and particularly by ATP and ADP, but not by AMP (Konopka, 1978), certainly implicate the importance of iron–nucleotide complexes in vivo. It is also extremely interesting that Hochstein et al. (1964) showed that lipid peroxidation of rat liver microsomes was dramatically enhanced by Fe–nucleotide complexes, particularly the di- and triphosphate nucleotide complexes, but not by AMP.

Also, in regard to damage induced by Fe(II)–small molecule complexes, it is interesting to note that Fe(II)–bleomycin complexes cause DNA strand breaks, possibly by an oxygen free radical intermediate [see Lown & Sim (1977) and Burger et al. (1981a), for example]. The Fe(III)–bleomycin complex has been characterized by EPR (Burger et al., 1981b). We have used their techniques to investigate if an EPR-detectable Fe(III)–ADP complex exists at 77 K, but no discrete EPR signals were observed.

The exact molecular arrangement of the Fe(II)–nucleotide complex(es) which catalyzes OH formation from H_2O_2 is not known. Metal ion–nucleotide complexes have been studied extensively, but very little is known specifically about Fe–nucleotide complexes. This is probably because of the paramagnetism of Fe(III) and the instability of Fe(II). Most extensive studies have been done with Cr(III)–ATP and Co(III)NH_4–ATP complexes (Merritt et al., 1978; Dunaway-Mariano & Cleland, 1980). Since Co(III) and Cr(III) have somewhat similar electronic configurations as Fe, these studies are probably quite relevant. In general, Cr(III) can form monodentate, bidentate, and tridentate complexes with ATP. These tend to be stable in mild acid but are hydrolyzed at neutral pH (Dunaway-Mariano & Cleland, 1980). It may be relevant to the present study to note that the bidentate complex of Cr(III)–ATP involves ligation to the β- and γ-phosphate ester oxygens rather than to the α-phosphate oxygen (Dunaway-Mariano & Cleland, 1980). The β,γ-bidentate Mg-ATP chelate is considered the substrate which binds to creatine kinase, yielding the α,β-Mg–ADP complex as the product (Burgers & Eckstein, 1980). It is only possible to speculate that Fe(II) may ligate with ATP and ADP in a similar fashion, and possibly these are the catalytic complexes responsible for OH formation from H_2O_2.

An examination of the differences between the amount of OH produced as a function of ADP vs. ATP concentration indicates that the Fe(II) complex with ATP is either more

stable or catalytically more active per se than the complex with ADP. But in contrast to ADP and ATP, AMP does not form a catalytically active complex. A preliminary examination of the ability of the ADP–Fe(II) complex to remain active in catalyzing OH formation from H_2O_2 has shown that it is nearly completely inactive after 10 min at room temperature. In view of the substantially reduced activity of the Fe(III)–nucleotide complexes and the results demonstrating the enhanced reduction in activity due to dissolved O_2, we speculate that the nucleotides hold Fe(II) in a configuration such that their oxidation to Fe(III) is less rapid and also prevent Fe(II), as well as Fe(III), from becoming ligated with buffer anions which are inactive in the catalysis of OH from H_2O_2.

The results with ascorbate emphasize the fact that Fe(III)–nucleotide complex can be reduced to the ferrous complex by a biological reductant which is then effective in catalyzing OH formation from H_2O_2. This work also strengthens the interpretation that it is the ferrous complex which is the catalytically active unit. This appears to be of high biological significance in oxidative damage due to oxygen free radicals.

Acknowledgments

The excellent secretarial assistance of E. Miser and Anita Hill is acknowledged.

Registry No. ADP, 58-64-0; ATP, 56-65-5; AMP, 61-19-8; Fe^{2+}, 15438-31-0; Fe^{3+}, 20074-52-6; hydroxyl radical, 3352-57-6; H_2O_2, 7722-84-1; O_2, 7782-44-7; N_2, 7727-37-9.

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Proton Nuclear Magnetic Resonance Studies of Porcine Intestinal Calcium Binding Protein†

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ABSTRACT: ^1H nuclear magnetic resonance has been employed to study the environment of several proton nuclei (primarily those arising from aromatic residues) of the porcine intestinal calcium binding protein. An assignment for the single tyrosine (Tyr-16) residue has been made on the basis of laser photochemically induced dynamic nuclear polarization (CIDNP) and homonuclear decoupling experiments. pH titration studies have shown that the tyrosine pK_a is unusually high in the apoprotein and increases even further upon the addition of calcium. However, the observation of a CIDNP effect with this tyrosine in both the presence and absence of calcium indicates that it is solvent accessible and therefore exposed on the surface of the molecule. Under the conditions of these experiments, the protein was observed to bind calcium with a 2:1 stoichiometry, at a rate of exchange slow enough that the NMR spectra are in the slow-exchange limit. The presence of upfield shifted phenylalanine and methyl resonances in the apoprotein indicates that there is a well-defined tertiary structure in the absence of calcium.

High-affinity calcium-binding proteins (CaBP)[1] with molecular weights near 10000 have been found in the small intestines of several mammalian species (Kallfelz et al., 1967; Drescher & DeLuca, 1971; Hitchman & Harrison, 1972). Porcine intestinal calcium binding protein is a compact, globular protein (M_r 8799), whose amino acid sequence of 78 residues has recently been determined (Hofmann et al., 1979). An examination of the evolutionary relationships among

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[1] Abbreviations: ICaBP, intestinal calcium binding protein; CaBP, calcium binding protein; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; ppm, parts per million; δ, chemical shift; CIDNP, chemically induced dynamic nuclear polarization; FMN, flavin mononucleotide; TnC, troponin C; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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