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  16. The four-coordinate structure is a direct band-gap semiconductor with an electronic absorbance peak in the visible spectrum, whereas the six-coordinate structure is an indirect band-gap semiconductor and has a featureless spectrum [see also (25)]. Light from a tungsten-halogen source was sent through a sample loaded in the DAC. The transmitted light was directed into a spectrometer and detected with a liquid N<sub>2</sub>-cooled charge-coupled device camera. Static temperature control to 510 K was achieved by placing the cell in a resistively heated ceramic oven and was measured with a thermocouple in contact with the diamond. Remote control of the pressure in the DAC by a computer-controlled stepper motor made it possible to measure relatively fast transition times on the order of seconds.
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## Antibody Catalysis of the Oxidation of Water

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Recently we reported that antibodies can generate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) from singlet molecular oxygen (<sup>1</sup>O<sub>2</sub>\*). We now show that this process is catalytic, and we identify the electron source for a quasi-unlimited generation of H<sub>2</sub>O<sub>2</sub>. Antibodies produce up to 500 mole equivalents of H<sub>2</sub>O<sub>2</sub> from <sup>1</sup>O<sub>2</sub>\*, without a reduction in rate, and we have excluded metals or Cl<sup>-</sup> as the electron source. On the basis of isotope incorporation experiments and kinetic data, we propose that antibodies use H<sub>2</sub>O as an electron source, facilitating its addition to <sup>1</sup>O<sub>2</sub>\* to form H<sub>2</sub>O<sub>3</sub> as the first intermediate in a reaction cascade that eventually leads to H<sub>2</sub>O<sub>2</sub>. X-ray crystallographic studies with xenon point to putative conserved oxygen binding sites within the antibody fold where this chemistry could be initiated. Our findings suggest a protective function of immunoglobulins against <sup>1</sup>O<sub>2</sub>\* and raise the question of whether the need to detoxify <sup>1</sup>O<sub>2</sub>\* has played a decisive role in the evolution of the immunoglobulin fold.

Antibodies, regardless of source or antigenic specificity, generate H<sub>2</sub>O<sub>2</sub> from <sup>1</sup>O<sub>2</sub>\*, thereby potentially aligning recognition and killing within the same molecule (1). Given the potential chemical and biological importance of this observation, the mechanistic basis of this process and its structural location within the antibody have been investigated. Together these studies reveal that antibodies, in contrast to other proteins, may catalyze an unprecedented set of chemical reactions between water and <sup>1</sup>O<sub>2</sub>\*.

Long-term ultraviolet (UV) irradiation studies reveal that antibody-mediated H<sub>2</sub>O<sub>2</sub> production is much more efficient than for non-immunoglobulin proteins (Fig. 1A). Typically antibodies exhibit linearity in H<sub>2</sub>O<sub>2</sub> formation for up to 40 mole equivalents of H<sub>2</sub>O<sub>2</sub> before the rate begins to decline asymptotically (Fig. 1B). Non-immunoglobulin proteins display a short burst of H<sub>2</sub>O<sub>2</sub> production followed by quenching as photo-oxidation

occurs (Fig. 1A). Also, antibodies can resume photoproduction of H<sub>2</sub>O<sub>2</sub> at the same initial rate if H<sub>2</sub>O<sub>2</sub> is removed by catalase (Fig. 1C). Thus, H<sub>2</sub>O<sub>2</sub> reversibly inhibits its own formation. The apparent median inhibitory concentration (IC<sub>50</sub>) was estimated as 225 μM (Fig. 1E). Antibody-mediated photoproduction of H<sub>2</sub>O<sub>2</sub> can also be saturated with molecular oxygen (apparent Michaelis-Menten constant for oxygen = 187 μM) (1), which, when allied with the H<sub>2</sub>O<sub>2</sub> inhibition aspect, suggests a binding site process.

Even after 10 cycles of UV irradiation followed by addition and removal of catalase (which generates ~500 mole equivalents of H<sub>2</sub>O<sub>2</sub>), only a slight reduction (5%) is seen in the initial rate. Beside antibodies, the only other protein that we have found thus far to generate H<sub>2</sub>O<sub>2</sub> catalytically is the αβ T cell receptor (αβTCR) (Fig. 1D), which shares a similar arrangement of its immunoglobulin fold domains with antibodies (2). However, possession of this structural motif does not necessarily confer an H<sub>2</sub>O<sub>2</sub>-generating ability on proteins; β<sub>2</sub>-microglobulin, although a member of the immunoglobulin superfamily (3), does not generate H<sub>2</sub>O<sub>2</sub>.

The antibody structure is remarkably inert to the oxidizing effects of H<sub>2</sub>O<sub>2</sub>. SDS-polyacrylamide gel electrophoresis of antibody samples after UV irradiation under standard conditions for 8 hours revealed no significant fragmenta-

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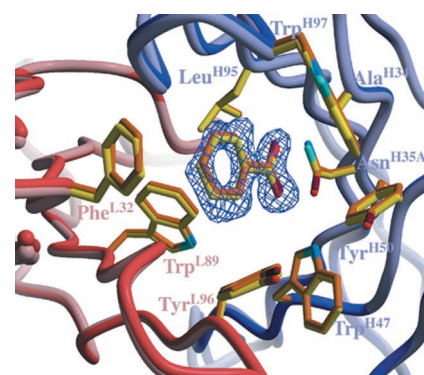
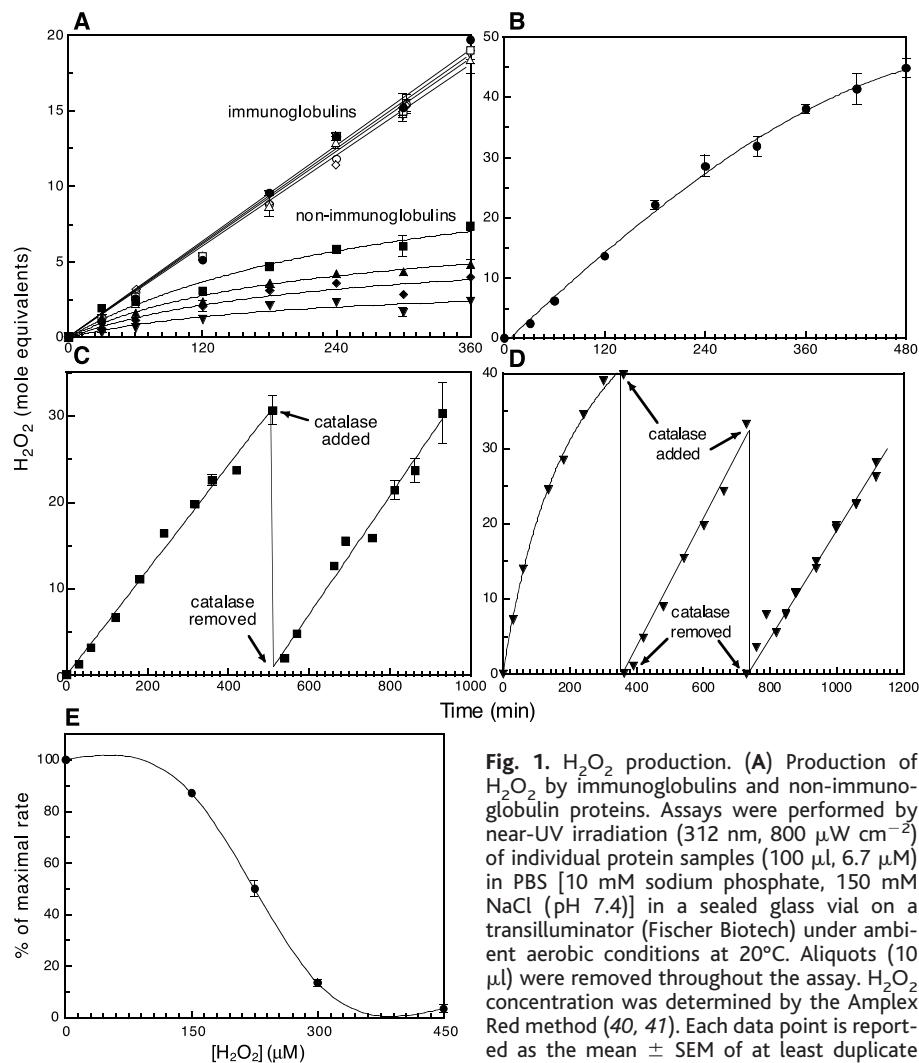
tion or agglomeration of the antibody. Also, the native and H<sub>2</sub>O<sub>2</sub>-treated structures of murine Fab 4C6 (4, 5) are superimposable at the level of side-chain positions, reinforcing the evidence of stability of the antibody fold in the presence of H<sub>2</sub>O<sub>2</sub> (Fig. 2).

The photoactivity of the antibody appears to be driven through tryptophan (Trp) absorbance. An action spectrum of the antibody-mediated

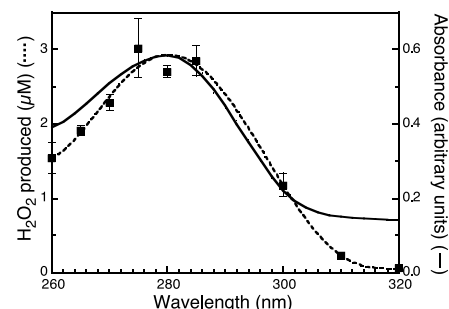
photoproduction of H<sub>2</sub>O<sub>2</sub> and the corresponding absorbance spectrum of the antibody protein for wavelengths from 260 to 320 nm are virtually superimposable (Fig. 3). The maximal efficiency of H<sub>2</sub>O<sub>2</sub> production occurs at the same wavelength as the UV absorbance maxima of Trp in proteins (~280 nm). We probed the efficiency of H<sub>2</sub>O<sub>2</sub> production by horse immunoglobulin G (IgG) as a function of the

efficiency of <sup>1</sup>O<sub>2</sub>\* formation via <sup>3</sup>O<sub>2</sub> sensitization with hematoporphyrin IX [quantum yield of singlet oxygen formation ( $\phi_{\Delta}$ ) = 0.22 in phosphate buffer (pH 7.0) and visible light (6, 7)]. For every 275 ± 25 mole equivalents of <sup>1</sup>O<sub>2</sub>\* generated by sensitization, 1 mole equivalent of H<sub>2</sub>O<sub>2</sub> was generated by the antibody molecule.

The conversion of <sup>1</sup>O<sub>2</sub>\* to H<sub>2</sub>O<sub>2</sub> requires two mole equivalents of electrons, and we have generated >500 equivalents of H<sub>2</sub>O<sub>2</sub> per equivalent of antibody molecule with no notable reduction in rate. Thus, the ultimate electron source clearly cannot be the antibody itself. Both as an individual amino acid and as a constituent of proteins, Trp is particularly sen-



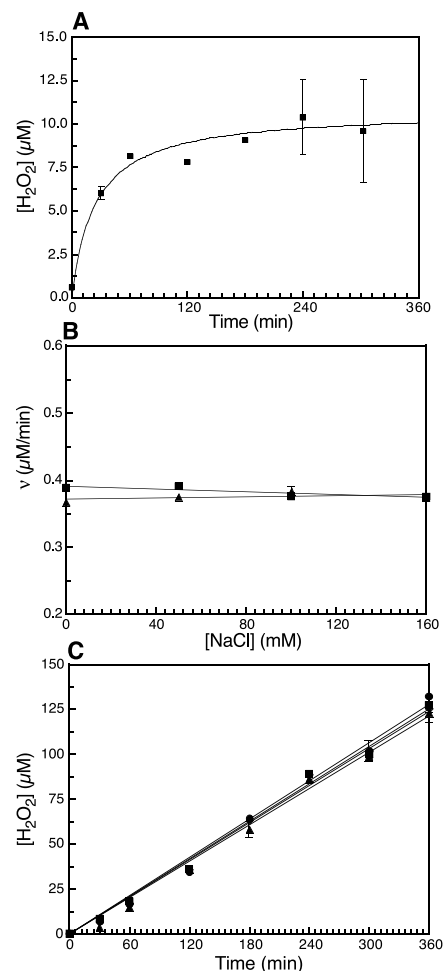
**Fig. 2.** Superposition of the 4C6 combining site with and without H<sub>2</sub>O<sub>2</sub> demonstrates that even the side-chain conformations within the binding site are preserved (light- and dark-colored side chains, pink for the light chains and blue for the heavy chains, correspond to + and - H<sub>2</sub>O<sub>2</sub>, respectively). Moreover, clear electron density for a benzoic acid ligand underscores that the binding properties of Fab 4C6 remain unaltered in 3 mM H<sub>2</sub>O<sub>2</sub>. The electron density map is a 2F<sub>obs</sub> - F<sub>calc</sub> σ-weighted map contoured at 1.5σ. (Figure was generated with Bobscript.) Details of this structure will be presented elsewhere.



**Fig. 3.** Absorbance spectrum (—) and action spectrum (—■—) of horse polyIgG between 260 and 320 nm. The absorbance spectrum was measured on a diode array HP8452A spectrophotometer (maximum absorbance, 280 nm). The action spectrum was measured by placing an antibody solution [6.7 μM in PBS (pH 7.4)] in a quartz tube in the light beam produced by a xenon arc lamp and monochromator of an SLM spectrofluorimeter for 1 hour. H<sub>2</sub>O<sub>2</sub> concentration was measured by the Amplex Red assay (40, 41).

polyIgG; □, sheep polyIgG; ▽, murine mIgG (WD1-6G6); △, human polyIgM; ◇, murine mIgG (92H2); ■, β-galactosidase (β-gal); ▲, chick ovalbumin; ▼, α-lactalbumin; ◆, bovine serum albumin]. (B) Long-term production of H<sub>2</sub>O<sub>2</sub> by sheep polyIgG (6.7 μM, 200 μl). Near-UV irradiation for 8 hours in PBS in a sealed well of a 96-well quartz plate, H<sub>2</sub>O<sub>2</sub> concentration was measured as described in (A). (C) A solution of murine mIgG PCP21H3 (6.7 μM, 200 μl) was irradiated in PBS in a sealed well of a 96-well quartz plate for 510 min. The H<sub>2</sub>O<sub>2</sub> was assayed by the Amplex Red assay and then destroyed by addition of catalase (10 mg, 288 mU) immobilized on Eupergit C. The catalase was removed by filtration, and the antibody solution was reirradiated for 420 min; rate (0 to 510 min) = 0.368 μM min<sup>-1</sup> (r<sup>2</sup> = 0.998); rate (511 to 930 min) = 0.398 μM min<sup>-1</sup> (r<sup>2</sup> = 0.987). This profile of continued linear production of H<sub>2</sub>O<sub>2</sub> after catalase-mediated destruction of H<sub>2</sub>O<sub>2</sub> is conserved for all antibodies assayed. (D) A solution of TCRαβ (6.7 μM, 200 μl) was irradiated as described in (C) for periods of 360, 367, and 389 min. The H<sub>2</sub>O<sub>2</sub> generated during each irradiation was assayed and destroyed as described in (C). The curvature in the progress curve above 30 mole equivalents conforms to the expected inhibition by H<sub>2</sub>O<sub>2</sub> (see below); rate (361 to 727 min) = 0.427 μM min<sup>-1</sup> (r<sup>2</sup> = 0.987); rate (728 to 1117 min) = 0.386 μM min<sup>-1</sup> (r<sup>2</sup> = 0.991). (E) Determination of IC<sub>50</sub> of H<sub>2</sub>O<sub>2</sub> on the photoproduction of H<sub>2</sub>O<sub>2</sub> by horse polyIgG. A solution of horse IgG (6.7 μM) was incubated with varying concentrations of H<sub>2</sub>O<sub>2</sub> (0 to 450 μM), and the initial rate of H<sub>2</sub>O<sub>2</sub> formation was measured as described in (A). The graph is a plot of rate of H<sub>2</sub>O<sub>2</sub> formation versus H<sub>2</sub>O<sub>2</sub> concentration and reveals an IC<sub>50</sub> of 225 μM.

sitive to near-UV irradiation (300 to 375 nm) under aerobic conditions, owing to its conversion to *N'*-formylkynurenine (NFK), which is a particularly effective near-UV ( $\lambda_{\max} = 320$



**Fig. 4.** (A) Production of H<sub>2</sub>O<sub>2</sub> by Trp (20 μM). The conditions and assay procedures were as described in Fig. 1A. (B) Effect of [Cl<sup>-</sup>] on antibody-mediated photoproduction of H<sub>2</sub>O<sub>2</sub>. A solution of sheep polyIgG (■, 6.7 μM, 200 μl) or horse polyIgG (▲, 6.7 μM, 200 μl) in PB (pH 7.4) was lyophilized to dryness and then dissolved in either deionized water or NaCl (aq.) such that the final [Cl<sup>-</sup>] was 0 to 160 mM. The samples were then irradiated, in duplicate, in sealed glass vials on a transilluminator (800 μW cm<sup>-2</sup>) under ambient aerobic conditions at 20°C. Aliquots (10 μl) were removed throughout the assay; H<sub>2</sub>O<sub>2</sub> concentration was determined by the Amplex Red assay (40, 41). The rate of H<sub>2</sub>O<sub>2</sub> formation, *v*, is plotted as the mean ± SEM versus [NaCl] for each antibody sample. (C) Effect of dialysis into EDTA-containing buffers on antibody-mediated photoproduction of H<sub>2</sub>O<sub>2</sub>. The photoproduction of H<sub>2</sub>O<sub>2</sub> by two antibody preparations, mouse mlgG PCP21H3 and horse polyIgG, were compared before and after dialysis into PBS containing EDTA (20 mM). The conditions and assay procedures were as described in Fig. 1A. Each data point is reported as the mean ± SEM of at least duplicate measurements (●, murine mlgG PCP21H3 before dialysis; ■, murine mlgG PCP21H3 after dialysis; ▲, horse polyIgG before dialysis; ◆, horse polyIgG after dialysis).

nm) photosensitizer (8). However, photo-oxidation of Trp (the free amino acid) is accompanied by substoichiometric production of H<sub>2</sub>O<sub>2</sub> (~0.5 mole equivalents) during near-UV irradiation (Fig. 4A) (9), and the most efficient non-immunoglobulin protein at H<sub>2</sub>O<sub>2</sub> photoproduction, β-galactosidase, generates only 7 mole equivalents of H<sub>2</sub>O<sub>2</sub> from its 39 Trp residues (10) (Fig. 1A). Even if every photo-oxidizable residue (Trp, Tyr, Cys, Met, and His) were consumed, this could not account for 500 mole equivalents of H<sub>2</sub>O<sub>2</sub> (7).

The next most likely source is Cl<sup>-</sup>, which is a suitable electron source for photoproduction of H<sub>2</sub>O<sub>2</sub> via a triplet-excited state of an anthraquinone (11). We thus investigated the potential of Cl<sup>-</sup> [present at 150 mM in phosphate-buffered saline (PBS)] as a reducing equivalent. However, the rate of H<sub>2</sub>O<sub>2</sub> production by immunoglobulins was independent of [Cl<sup>-</sup>] in the range 0 to 160 mM (Fig. 4B).

We also considered the possible role of metal ions. Although such ions could hardly be sufficiently abundant in antibodies to serve as an electron source, trace amounts of them might play a central role as catalytic redox centers. The following experiments allowed us to rule out the implication of trace metals in this process: (i) The rate of antibody-mediated photoproduction of H<sub>2</sub>O<sub>2</sub> is unchanged before and after exhaustive dialysis of antibody samples with EDTA-containing buffer (Fig. 4C). (ii) After EDTA treatment of antibody samples, inductively coupled plasma-atomic emission spectroscopy (ICP-AES) reveals the presence of remaining trace metal ions in amounts far less than 1 part per million (7). (iii) For a trace metal to be implicated in this reaction, it must be common to all antibodies because all antibodies assayed have this intrinsic ability. It is generally accepted that metal binding is not an implicit feature of antibodies; this idea is consistent with our own analysis of antibody crystals as well as the ~300 antibody structures available in the Brookhaven database.

All of our observations thus far pointed toward an electron source that does not deactivate the protein catalyst, could account for the high turnover numbers, and hence is quasi-unlimited. Our attention thus turned to a broader consideration of the chemical potential of <sup>1</sup>O<sub>2</sub>\*

The known chemistry of <sup>1</sup>O<sub>2</sub>\* (12) can be conceptualized as the chemistry of the super-electrophile “dioxa-ethene.” So we considered that a molecule of water may, in the presence of an antibody, add as a nucleophile to <sup>1</sup>O<sub>2</sub>\* and form H<sub>2</sub>O<sub>3</sub> as an intermediate. Water, in becoming oxidized to H<sub>2</sub>O<sub>2</sub>, would fulfill the role of the electron source.

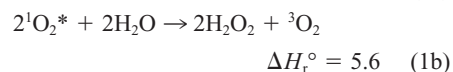
Isotope experiments were undertaken to determine the source of oxygen found in the H<sub>2</sub>O<sub>2</sub>. Contents of <sup>16</sup>O/<sup>18</sup>O in H<sub>2</sub>O<sub>2</sub> were measured by modification of a stan-

dard H<sub>2</sub>O<sub>2</sub> detection method: reduction with tris(carboxyethyl)phosphine (TCEP) (13) followed by mass-spectral analysis of the corresponding phosphine oxides (Fig. 5).

In the presence of oxygen, UV irradiation of antibodies leads to oxygen incorporation from water into H<sub>2</sub>O<sub>2</sub> (7). The relative abundance of the <sup>16</sup>O/<sup>18</sup>O ratio observed in the mass spectra of the phosphine oxide after irradiation of sheep polyclonal IgG (polyIgG) under conditions of saturating <sup>16</sup>O<sub>2</sub> concentration in a solution of H<sub>2</sub><sup>18</sup>O (98% <sup>18</sup>O) phosphate buffer (PB) was (2.2 ± 0.2):1 (Fig. 5A) (7). When the converse experiment was performed with an <sup>18</sup>O-enriched molecular oxygen mixture (90% <sup>18</sup>O) in H<sub>2</sub><sup>16</sup>O PB, the reverse ratio [1:(2.0 ± 0.2)] was observed (Fig. 5B) (14). These ratios exhibit good reproducibility (±10%, *n* = 10) (15) and were found for all antibodies studied (16).

The following control experiments were performed. First, under conditions of <sup>16</sup>O<sub>2</sub> and H<sub>2</sub><sup>16</sup>O, irradiation of horse polyIgG generates H<sub>2</sub><sup>16</sup>O<sub>2</sub> (Fig. 5C). No incorporation of <sup>18</sup>O occurs when H<sub>2</sub><sup>16</sup>O<sub>2</sub> (400 μM in PB, pH 7.0) itself is irradiated for 4 hours in H<sub>2</sub><sup>18</sup>O. Thus, <sup>18</sup>O incorporation into H<sub>2</sub>O<sub>2</sub> does not occur either by an acid-catalyzed exchange with water or by a mechanism that involves homolytic cleavage of H<sub>2</sub><sup>16</sup>O<sub>2</sub> and recombination with H<sup>18</sup>O• from water. To investigate the possibility that antibodies may catalyze both the production of H<sub>2</sub><sup>16</sup>O<sub>2</sub> and its acid-catalyzed exchange with H<sub>2</sub><sup>18</sup>O, we determined the isotopic exchange of H<sub>2</sub><sup>16</sup>O<sub>2</sub> (200 μM) in H<sub>2</sub><sup>18</sup>O (98% <sup>18</sup>O) PB in the presence of sheep polyIgG (6.7 μM) after UV irradiation under an inert atmosphere. Only a trace of incorporation of <sup>18</sup>O into H<sub>2</sub><sup>16</sup>O<sub>2</sub> (<1%) was observed (Fig. 5D) (17).

The thermodynamic balance between reactants and products for the oxidation of H<sub>2</sub>O by <sup>1</sup>O<sub>2</sub>\* (heat of reaction  $\Delta H_r^\circ = +28.1$  kcal/mol, Eq. 1a) (18) demands a stoichiometry in which more than one molecule of <sup>1</sup>O<sub>2</sub>\* must participate per molecule of oxidized water during its conversion into two molecules of H<sub>2</sub>O<sub>2</sub>. This stoichiometry assumes that no further light energy apart from that involved in the production of singlet from triplet oxygen is participating in the process. Qualitative chemical reasoning on hypothetical mechanistic pathways, together with thermodynamic considerations, makes the overall stoichiometries likely to be those shown in Eqs. 1b or 1c (heats of formation  $\Delta H_f^\circ$  are reported in kcal/mol):



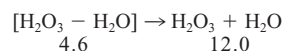
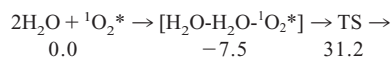
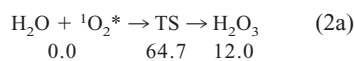
A recent report of a transition metal-catalyzed conversion of <sup>1</sup>O<sub>2</sub> and water into H<sub>2</sub>O<sub>2</sub> via a tellurium-mediated redox process (19) provides



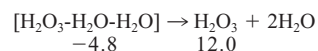
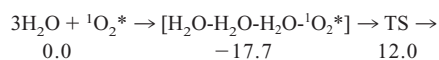
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experimental evidence for a process in which  $^1\text{O}_2^*$  and  $\text{H}_2\text{O}$  can be converted into  $\text{H}_2\text{O}_2$ . Hence, the energetic demands of this process can be overcome. At the heart of our considerations of a mechanism for the antibody-mediated photo-oxidation process is the hypothesis that addition of a water molecule to a molecule of  $^1\text{O}_2^*$  forms  $\text{H}_2\text{O}_3$  as the first intermediate on the way to  $\text{H}_2\text{O}_2$ . The antibody's function as a catalyst would have to be the supply of a specific molecular environment that would stabilize this critical intermediate relative to its reversible formation and/or would accelerate the consumption of the intermediate by channeling its conversion to  $\text{H}_2\text{O}_2$ . An essential feature of such an environment might consist of a special constellation of organized water molecules at an active site conditioned by an antibody-specific surrounding.

Although  $\text{H}_2\text{O}_3$  has not yet been detected in biological systems, its chemistry *in vivo* has been a source of considerable speculation, and its *in vitro* properties have been the subject of numerous experimental and theoretical treatments (20–27). Koller and Plesnicar have shown that  $\text{H}_2\text{O}_3$  reductively generated from ozone decomposes into  $\text{H}_2\text{O}$  and  $^1\text{O}_2^*$  in a process catalyzed by a water molecule (26). Applying the principle of microscopic reversibility, we surmised that one or more molecules of water should also catalyze the reverse reaction. To delineate plausible reaction routes and energetics of such a process, we used first-principles quantum chemical (QC) methods [B3LYP (7)]:



(2b)



(2c)

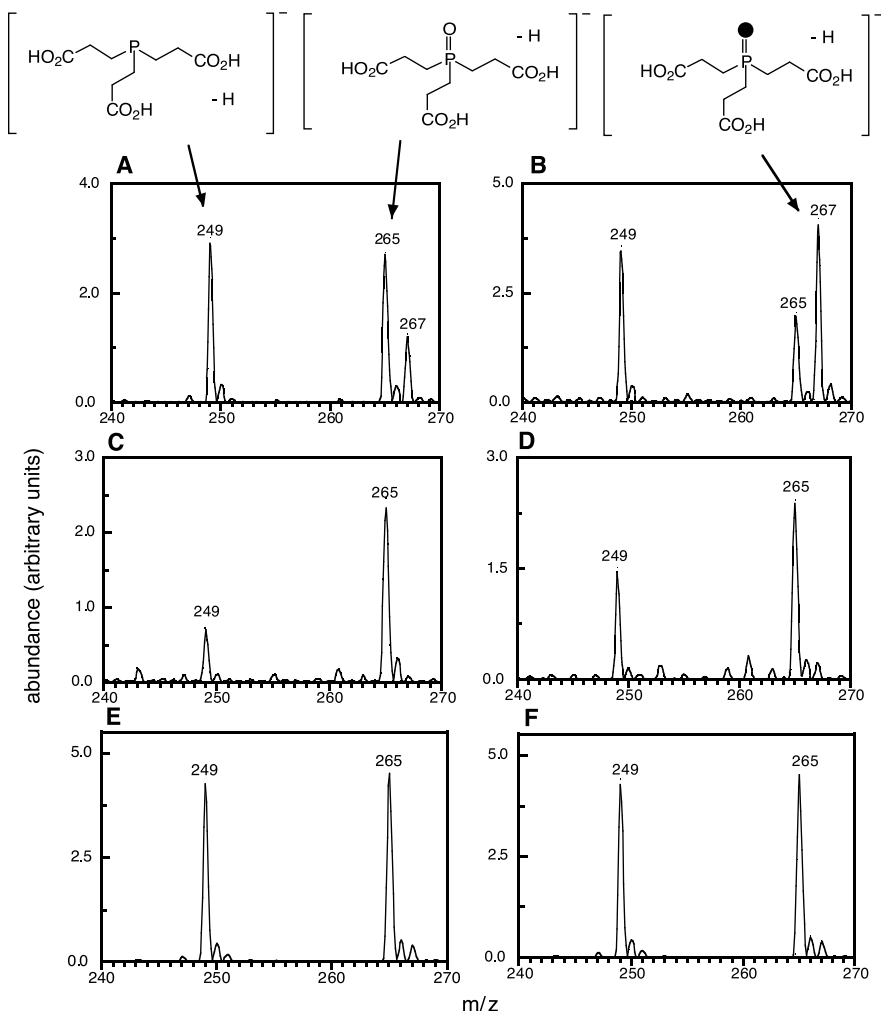
In these equations, all energetics are in kcal/mol. The direct reaction of water and  $^1\text{O}_2^*$  to give  $\text{H}_2\text{O}_3$  is quite unfavorable, with an activation barrier of 64.7 kcal/mol (Eq. 2a). However, with the addition of a second or third water molecule, we find a concerted process that decreases the activation barrier to 31.2 kcal/mol and 12.0 kcal/mol, respectively. Indeed, these additional waters play a catalytic role (in Eq. 2b, the H of the second water goes to the product HOOH, simultaneous with the H of the first water replacing it). Note that the reverse reaction in Eqs. 2b and 2c has a barrier of

only 19.2 kcal/mol or 0 kcal/mol, respectively, which suggests that  $\text{H}_2\text{O}_3$  is not stable in bulk water or water-rich systems. Thus, we expect that the best site within the antibody structure for producing and using  $\text{H}_2\text{O}_3$  would be one in which there are localized waters and water dimers next to hydrophobic regions without such waters.

We note that a 2.2:1  $^{16}\text{O}/^{18}\text{O}$  incorporation ratio would coincide exactly with the value predicted for certain mechanisms in which two molecules of  $^1\text{O}_2^*$  and two molecules of  $\text{H}_2\text{O}$  are transformed into two molecules of  $\text{H}_2\text{O}_2$  and one molecule of  $\text{O}_2$  (which would have to be  $^3\text{O}_2$  for thermodynamic reasons). An example is a second-order nucleophilic substitution ( $\text{S}_{\text{N}}2$ -type disproportionation) of two molecules of  $\text{H}_2\text{O}_3$  into  $\text{H}_2\text{O}_4$  and  $\text{H}_2\text{O}_2$ , followed by the

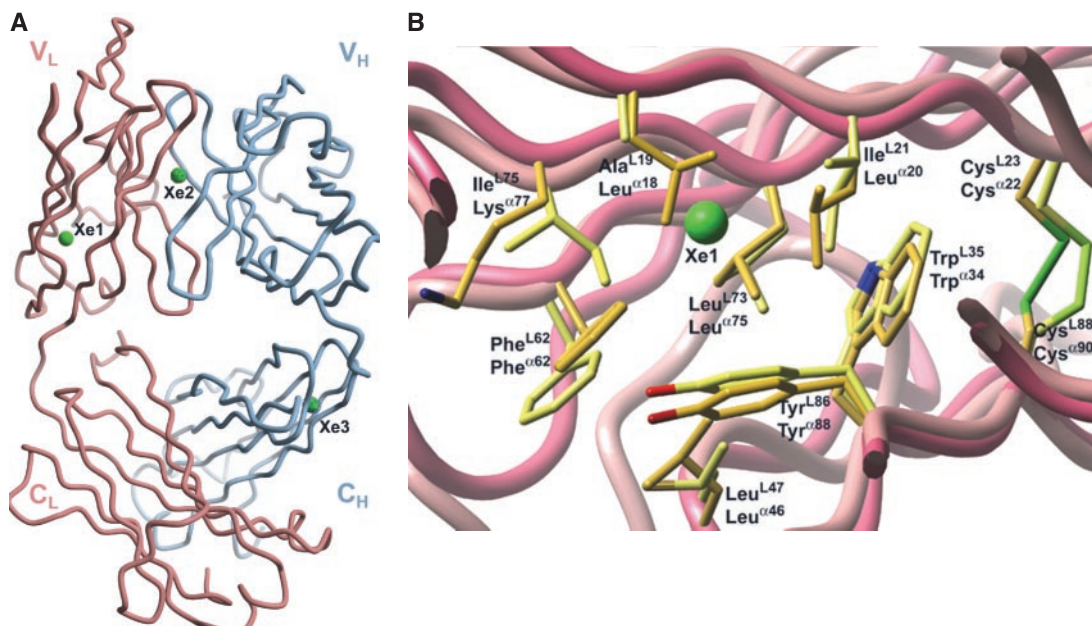
decomposition of the former into  $\text{H}_2\text{O}_2$  and  $^3\text{O}_2$  (28). Although our experimental evidence leads us to a hypothesis for the oxidation of water via  $\text{H}_2\text{O}_3$ , we have not discounted other mechanistic routes that may depend on a concert of events that are unique to antibodies.

Given the conserved ability of antibodies (regardless of origin or antigen specificity) and of the  $\alpha\beta\text{TCR}$  to mediate this reaction, x-ray structural studies were instigated to search for a possible conserved reaction site within these immunoglobulin fold proteins. A key constraint for any potential locus is that molecular oxygen (either  $^1\text{O}_2^*$  or  $^3\text{O}_2$  with a potential sensitizing residue, preferably Trp, in proximity) and water must be able to colocalize, and the transition states and intermediates along the pathway must be stabilized either within the site or in



**Fig. 5.** Electrospray ionization (negative polarity) mass spectra of TCEP [(M-H)<sup>-</sup> 249] and its oxide [(M-H)<sup>-</sup> 265 ( $^{16}\text{O}$ ) and (M-H)<sup>-</sup> 267 ( $^{18}\text{O}$ )] produced by oxidation with  $\text{H}_2\text{O}_2$  [see (7) for assay conditions]. (A) After irradiation of sheep polyIgG (6.7  $\mu\text{M}$ ) under  $^{16}\text{O}_2$  aerobic conditions in  $\text{H}_2^{18}\text{O}$  (98%  $^{18}\text{O}$ ) PB. (B) After irradiation of sheep polyIgG (6.7  $\mu\text{M}$ ) under enriched  $^{18}\text{O}_2$  (90%  $^{18}\text{O}$ ) aerobic conditions in  $\text{H}_2^{16}\text{O}$  PB (16). (C) After irradiation of sheep polyIgG under  $^{16}\text{O}_2$  aerobic concentration in  $\text{H}_2^{16}\text{O}$  PB. In this assay,  $\text{H}_2^{16}\text{O}$  replaced  $\text{H}_2^{18}\text{O}$ . (D) After irradiation of sheep polyIgG (6.7  $\mu\text{M}$ ) and  $\text{H}_2^{16}\text{O}_2$  (200  $\mu\text{M}$ ) under anaerobic (degassed under argon) conditions in  $\text{H}_2^{18}\text{O}$  PB for 8 hours at 20°C. (E) After irradiation of 3-methylindole (500  $\mu\text{M}$ ) under  $^{16}\text{O}_2$  aerobic conditions in  $\text{H}_2^{18}\text{O}$  PB. Size-exclusion filtration was not performed because of the low molecular weight of 3-methylindole. TCEP was added to the 3-methylindole-containing PB solution. (F) After irradiation of  $\beta$ -gal (50  $\mu\text{M}$ ) under  $^{16}\text{O}_2$  aerobic conditions in  $\text{H}_2^{18}\text{O}$  PB.

**Fig. 6.** The Xe binding sites in antibody 4C6 (7). **(A)** Standard side view of the  $C_\alpha$  trace of Fab 4C6, with the light chain in pink and the heavy chain in blue. Three bound xenon atoms (green) are shown with the initial  $F_{\text{obs}} - F_{\text{calc}}$  electron density map contoured at  $5\sigma$ . **(B)** Overlay of Fab 4C6 and the 2C  $\alpha\beta$ TCR (1TCR) around the conserved Xe1 site. The backbone  $C_\alpha$  trace of  $V_L$  (pink) and side chains (yellow) and the corresponding  $V_\alpha$  of the 2C  $\alpha\beta$ TCR (red and gold) are superimposed. (Figure was generated with Insight 2000.)



close proximity. Xenon gas was used as a heavy-atom tracer to locate cavities within the murine monoclonal antibody 4C6 (5, 7) that may be accessible to  $O_2$  (29–31). Three xenon sites (Xe1, Xe2, and Xe3) were identified (Fig. 6A), and all occupy hydrophobic cavities, as observed in other Xe-binding sites in proteins (32, 33). Superposition of the refined native and Xe-derivatized structures shows that, aside from addition of Xe, there is little discernible change in the protein backbone or side-chain conformation or in the location of bound water molecules.

The Xe1 site is conserved in all of the antibodies we studied and the  $\alpha\beta$ TCR (Fig. 6B). Xe1 is in the middle of a highly conserved region between the  $\beta$  sheets of  $V_L$  (the variable region of immunoglobulin light chain), 7 Å from an invariant Trp. The Xe1 site is sandwiched between the two  $\beta$  sheets that constitute the immunoglobulin fold of the  $V_L$ , ~5 Å from the outside molecular surface. Xe2 sits at the base of the antigen binding pocket directly above several highly conserved residues that form the structurally conserved interface between the heavy and light chains of an antibody (Fig. 6A). The residues in the  $V_L V_H$  interface are primarily hydrophobic and include conserved aromatic side chains such as Trp<sup>H103</sup>.

The contacting side chains for Xe1 in Fab 4C6 are Ala<sup>L19</sup>, Ile<sup>L21</sup>, Leu<sup>L73</sup>, and Ile<sup>L75</sup>, which are highly conserved aliphatic side chains in all antibodies; only slight structural variation was observed in this region in all antibodies surveyed. Notably, several other highly conserved and invariant residues are in the immediate vicinity of this xenon site, including Trp<sup>L35</sup>, Phe<sup>L62</sup>, Tyr<sup>L86</sup>, Leu<sup>L104</sup>, and the disulfide bridge between Cys<sup>L23</sup> and Cys<sup>L88</sup>. Trp<sup>L35</sup> stacks against the disulfide bridge and is only 7 Å from the xenon atom. In this structural context, Trp<sup>L35</sup>

may be a putative molecular oxygen sensitizer, because it is the closest Trp to Xe1. Comparison with the 2C  $\alpha\beta$ TCR structure (3) and all available TCR sequences shows that this Xe1 hydrophobic pocket is also highly conserved in TCRs (Fig. 6B). Thus, the xenon experiments have identified at least one site that is both accessible to molecular oxygen and is in a conserved region ( $V_L$ ) in close proximity to an invariant Trp; an equivalent conserved site is also possible in the fold of  $V_H$  (34). Analysis of the sequence and structure around these sites shows that they are highly conserved in both antibodies and TCRs. This finding may provide a possible understanding of why the Ig fold in antibodies and the TCR can be involved in this unusual chemistry (35).

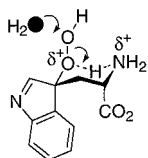
As discussed previously (1), antibody-catalyzed production of  $H_2O_2$  from  $^1O_2^*$  may participate in antibody-mediated cell killing by event-related production of  $H_2O_2$ . Alternatively, antibodies may function in defending an organism against  $^1O_2^*$ . This postulate would require the further processing of  $H_2O_2$  into water and  $^3O_2$  by catalase (36). Because catalase is known to be an ancient protein arising as far back as archaeobacteria (37), the question can be raised as to whether the structural element responsible for the catalytic destruction of  $^1O_2^*$  is equally ancient and considerably precedes what we know today as antibodies. Singlet oxygen may even have played a decisive role in the initiation of the evolution of the immunoglobulin fold. Thus, it makes sense to search among ancient aerobic organisms for proteins that can accomplish similar chemistry.

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14. In a typical experiment, a solution of sheep or horse polyIgG (6.7  $\mu$ M, 100  $\mu$ l) in PB (160 mM phosphate, pH 7.4) was degassed under an argon atmosphere for 30 min. This solution was then saturated with  $^{18}O_2$  (90%) and assayed as described (7).
15. The reproducibility of the  $O^{16}/O^{18}$  ratio from protein samples lyophilized together is reasonable ( $\pm 10\%$ ). However, problems with removing protein-bound water molecules during the lyophilization process means that the observed ratios can vary between samples from different lyophilization batches by as much as 2:1 to 4:1 (when lyophilizing from  $H_2^{16}O$ ). It is therefore imperative that rigorous lyophilization and degassing procedures are followed. In this regard, the  $^{18}O_2$  and  $H_2^{16}O$  experiments exhibit less inter-assay variability because of the ease of removing protein-bound oxygen molecules.
16. Antibodies from different species give similar ratios within the experimental constraints detailed in (15). Observed  $^{16}O:^{18}O$  ratios are as follows: WD1-6G6 mIgG (murine), 2.1:1; horse polyIgG, 2.2:1; sheep polyIgG, 2.2:1; EP2-19G2 mIgG (murine), 2.1:1; CH2-

- 5H7 mlgG (murine), 2.0:1; human polyIgG, 2.1:1. Ratios are based on means of duplicate determinations, except for horse polyIgG, which is the mean of 10 measurements (7).
17. Isotope experiments were also performed with  $\beta$ -galactosidase, the most efficient non-immunoglobulin protein at generating  $H_2O_2$  (see above), as well as 3-methylindole. In both cases, photo-oxidation led to negligible  $^{18}O$  incorporation into the  $H_2O_2$  (Fig. 5, E and F, respectively), illustrating the view that the indole ring itself and tryptophan residues in this protein are behaving simply as reductants of  $^1O_2^*$ . Irradiation of 3-methylindole generates  $H_2O_2$  that does not include oxygen incorporation from  $H_2^{18}O$ . The same experiment performed with Trp gives rise to exchange with a  $^{16}O/^{18}O$  ratio of 1.2:1. We attribute this result to the ammonium functionality acting as an intramolecular general acid that protonates the internal oxygen of a diastereomeric mixture of 3'-hydroperoxides. This process cannot account for the catalytic production of  $H_2O_2$  by antibodies because it is stoichiometric.
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34. The structure and sequence around the Xe1 site is almost exactly reproduced in the  $V_H$  domain by the pseudo-twofold rotation axis that relates  $V_L$  to  $V_H$ . Although we did not locate a Xe binding site in this domain,  $O_2$  could still access the corresponding cavity in  $V_H$ . The proposed heavy-chain Xe site may not have been found because the crystals were pressurized for only 2 min, due to Xe being too large compared to  $O_2$  for the corresponding cavity on the  $V_H$  side, or because of crystal packing. In other antibody experiments, Xe binding sites were found in only one of the two molecules of the

- asymmetric unit, which suggests that crystal packing can modulate access of Xe in crystals.
35. Human  $\beta_2$ -microglobulin, which does not generate  $H_2O_2$  (see above), does not have the same detailed structural characteristics that define the antibody Xe1 binding pocket, despite its overall immunoglobulin fold. Also,  $\beta_2$ -microglobulin does not contain the conserved Trp residue that occurs there in both antibodies and TCRs. If Trp<sup>335</sup> (antibodies) or Trp<sup>334</sup> (TCR) is the oxygen sensitizer, the lack of a corresponding Trp in  $\beta_2$ -microglobulin may relate to the finding that it does not catalyze the oxidation of water.
36. For such a protection mechanism to be effective, catalase must not generate  $^1O_2^*$  during  $H_2O_2$  destruction. Currently, there is conflicting evidence regarding  $^1O_2^*$  generation from catalase-mediated decomposition of  $H_2O_2$  [see (39)].
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41. Any concerns that the Amplex Red assay may be detecting protein-hydroperoxide derivatives in addition to  $H_2O_2$  have been discounted, because the apparent  $H_2O_2$  concentration measured using this method is independent of whether irradiated protein is removed from the sample (by size-exclusion filtration).
42. We thank members of the Scripps Research Institute mass spectroscopy facility, especially G. Suizdak and M. Sonderegger, for assistance with the isotope analysis; L. Teyton for the 2C TCR $\alpha\beta$ ; M. Pique for Kabat database analysis; K. Quon for ICP-AES antibody analyses; B. Zhou for mutagenesis studies; P. G. Schultz for helpful discussions; D. Datta, N. Vaidehi, R. P. Muller, and D. Chakraborty for stimulating discussions; and several Wilson lab members for help with data collection and processing, especially X. Daio. Supported by NIH grants GM43858 (K.D.J.), CA27489 (program project grant; K.D.J., I.A.W., R.A.L.), and HD 36385 (W.A.G.).

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## The Role of Atomic Ensembles in the Reactivity of Bimetallic Electrocatalysts

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Bimetallic electrodes are used in a number of electrochemical processes, but the role of particular arrangements of surface metal atoms (ensembles) has not been studied directly. We have evaluated the electrochemical/catalytic properties of defined atomic ensembles in atomically flat PdAu(111) electrodes with variable surface stoichiometry that were prepared by controlled electrodeposition on Au(111). These properties are derived from infrared spectroscopic and voltammetric data obtained for electrode surfaces for which the concentration and distribution of the respective metal atoms are determined in situ by atomic resolution scanning tunneling microscopy with chemical contrast. Palladium monomers are identified as the smallest ensemble ("critical ensemble") for carbon monoxide adsorption and oxidation, whereas hydrogen adsorption requires at least palladium dimers.

Electrocatalytic reactions are of central importance in electrochemistry and play a vital role in emerging technologies related to environmental and energy-related applications, such as fuel cells. The efficiency and selectivity of electrocatalytic processes can be substantially improved by replacing monometallic with bimetallic catalysts. For example, the standard Pt electrocatalysts in polymer electrolyte membrane fuel cells are now being replaced by PtRu and PtMo alloys. The

development of these bimetallic catalysts has been based primarily on empirical grounds. However, a detailed knowledge of the physical origins underlying the improvements in catalytic performance has been lacking so far.

Three explanations have been put forward for the higher activity of bimetallic catalysts: (i) Each metal component could promote different elementary reaction steps, leading to a "bifunctional mechanism" (1). (ii) Electronic effects resulting from interactions between the two metals could improve reactivity (2). (iii) The concept of geometric ensemble effects (specific groupings of surface atoms are required to serve as active sites), developed in heterogeneous gas-phase catalysis (3), has also been suggested for electrocatalysis (4). However, the direct experimental verification or quantitative assessment of the relative contributions of these effects has not been possible up to now. The lack of data on the local atomic arrangement at the surface, both for

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