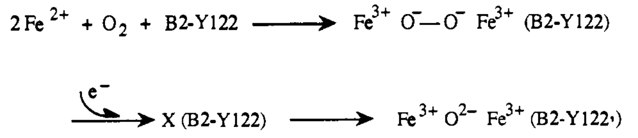
Scheme I



ions in solution.[13] The magnetic Mössbauer component must be associated with (X), since no EPR signal attributable to iron is observed other than the $g = 2.0$ ($S = {}^1/_2$) signal due to (X). This component can be interpreted as a superposition of two spectral components of equal intensity, each originating from an iron site (top of Figure 2A). It can be analyzed by the following spin Hamiltonian with $S = {}^1/_2$ (eq 1),

$$\hat{H} = \beta\vec{S}\cdot\tilde{\mathbf{g}}\cdot\vec{H} + \vec{S}\cdot\tilde{\mathrm{A}}\cdot\vec{I} + \frac{eQV_{zz}}{4}\left[I_z^2 - I(I+1)/3 + \frac{\eta}{3}(I_x^2 - I_y^2)\right] - g_n\beta_n\vec{H}\cdot\vec{I} \quad (1)$$

and using the following parameters: for site 1, $\Delta E_Q = -1.0$ mm/s,[14] $\eta = 0.5$, $\delta = 0.55$ mm/s, and $|A/g_n\beta_n| = 52$ T; for site 2, $\Delta E_Q = -1.1$ mm/s,[14] $\eta = 0.7$, $\delta = 0.45$ mm/s, and $|A/g_n\beta_n| = 24.5$ T. The solid line plotted over the experimental spectrum (Figure 2A) is the theoretical simulation resulting from this analysis. Good agreement between experiment and theory is observed. By using the same A values, the corresponding EPR spectrum can also be simulated. Again, excellent agreement is observed between experiment (Figure 1B) and theory (Figure 1C). This agreement establishes unambiguously the association of the magnetic Mössbauer component with the $g = 2.0$ EPR signal due to (X). The isomer shifts and the isotropic A values used in the simulations suggest that (X) is composed of two high-spin ferric ions ($S = {}^5/_2$). Since two $S = {}^5/_2$ ions cannot by themselves couple to form the observed $S = {}^1/_2$ system spin, a third constituent with half-integer spin is required. We propose that this constituent is either an oxygen radical or an amino acid radical, and we designate the intermediate, (X), a *diferric radical species*.

Having characterized this novel diferric radical species in B2-Y122F, a similar experiment was performed using B2-wt. The resulting Mössbauer spectrum (Figure 2B) is identical to that observed with B2-Y122F, except that the total amount of (X)/B2 is less (0.9 equiv compared to 1 equiv in B2-Y122F) and ~0.1 equiv of the product diferric cluster is also present. As alluded to above, (X) formed in B2-wt is kinetically competent to produce •Y122, resulting in the product diferric cluster.[10]

The detailed structure of the diferric radical intermediate, (X), remains to be established. A key question in this regard is the identity of the radical constituent. The observation of (X) with both B2-wt and B2-Y122F eliminates •Y122 as a candidate. Since the large ^{57}Fe hyperfine coupling observed in (X) suggests that the radical is ligated to either one or both ferric ions, the recent report of the X-ray structure of B2[15] defines defines the remaining possibilities. One possible candidate is an oxyl or hydroxyl radical derived either from O_2 or from H_2O. Experiments using $^{17}O_2$ and $H_2{}^{17}O$ to test this hypothesis are in progress.

Acknowledgment. We thank Mr. B. Pucket for the design and fabrication of the rapid freeze-quench Mössbauer cell.

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(14) The rapid-quench Mössbauer samples were frozen in isopentane which has a melting temperature of 112.7 K and is present in the packed samples. To obtain the ΔE_Q value for a paramagnetic iron center, the general practice is to increase the temperature until the electronic relaxation rate is fast in comparison with the nuclear precession time (10^{-7} s) and collapse the Mössbauer spectrum into a quadrupole doublet. However, at temperatures just below 112 K, the electronic relaxation rate of the diferric intermediate (X) is such that its Mössbauer spectrum is broad and featureless. Consequently, the value of ΔE_Q is chosen within the range for high-spin ferric ions and through comparison of a series of theoretical simulations with experimental data, which suggest a value of less than 1.5 mm/s.

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Photoelectrochemical Behavior of C_{60} Films

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Fullerene allotropes of carbon can now be produced in quantities suitable for conventional reaction and structural studies.[1] Of particular relevance from these advances is the rich electrochemical behavior (three successive, reversible reduction steps) that has been shown for both C_{60} and C_{70} molecules in solution[2,3] and the redox activity[4] of C_{60} films, CH_2Cl_2-cast onto microelectrodes. We have found that solvent-cast films of C_{60} on noble-metal electrodes show the photovoltaic response typical of n-type semiconductors in liquid junction cells. This search was initiated when experiments on sublimed films bridging metals indicated the existence of a photoconductive response linear in bias voltage and excitation intensity.[5] Photovoltaic behavior of organic molecular solids has an extensive history.[6] We demonstrate the photovoltaic character of C_{60} liquid junctions through voltammetry of cast film-rotating disk electrodes (RDE) under chopped light, measurements of photovoltage and photocurrent dependence on irradiance from an Ar laser, and by excitation spectra of the photocurrent response.

Solvent-cast films (from benzene solution) of C_{60} are quite insoluble in most nonaromatic organic solvents, and we have used acetonitrile solutions with 0.3 M tetra-*n*-butylammonium perchlorate [(TBA)ClO_4/MeCN] as supporting electrolyte in all cases. Jehoulet, Bard, and Wudl[4] have shown that their cast films have redox activity in MeCN solution when the TBA cation is present. Our results in the course of this work are consistent with their description of the hysteresis of the reduction-reoxidation of such films. Very significantly, for the photoelectrochemical behavior we demonstrate, the C_{60} electrode is stable to anodic reaction over a region of more than 2 V, up to +1.3 V vs the ferrocene/ferrocinium couple.[4] Thus, for n-type behavior, photooxidation of a redox species in solution has an ample potential window to compete successfully with substrate reaction, a relatively unusual situation for n-type semiconductors of solar-matched band gaps.[7,8]

Films of about 50-100 monolayers (estimated as in ref 4) were cast onto a Pt RDE of about 0.2-cm diameter, sheathed in Teflon (Pine Instrument Co.), from dilute (ca. 0.1 mM) benzene solution. Benzene was allowed to evaporate at ambient temperature leaving a pale yellow-brown film whose uniformity was not otherwise controlled. Variability from this simple procedure occurs only in the quantitative photocurrent sensitivity observed, not in any other effects described. The electrode, after coating, was run in 0.3 M (TBA)ClO_4/MeCN containing 1-100 mM iodide (as TBA salt).[9] No obvious changes occurred in the film during experi-

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(9) Electrodes were coated and handled in the ambient atmosphere with no noticeable deterioration. Electrochemical experiments were carried out with a three-electrode configuration; filmed working electrode, carbon-rod counter electrode, and saturated calomel (SCE) reference electrode. A Ag wire was substituted where noise pickup was a problem, as quasi-reference with SCE as standard. All electrolytes contained 0.3 M (TBA)ClO_4 in MeCN, with either 1 or 100 mM (TBA)I, as specified. A Pine Instrument RDE3 potentiostat was used in all controlled potential experiments. Rotation conditions were always 1600 rpm for the 0.196-cm^2 Pt disk.

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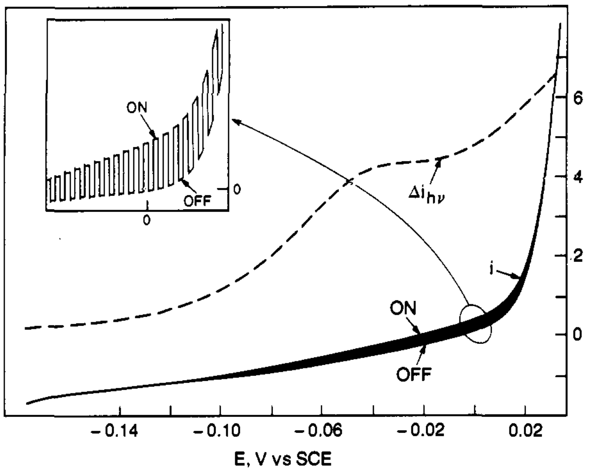

Figure 1. Controlled-potential scan (2 mV/s) under 14-Hz chopped, 30-mW Ar ion laser illumination(multiline) of C_{60} film on Pt RDE: (trace i) total current (corresponding to ordinate values shown); (dashed line) filtered and rectified 14-Hz component (arbitrary scale proportional to vertical on–off height of trace i). [(TBA)I] = 0.1 M.

ments, and the electrode could be removed, washed with MeCN, and reinserted with retention of most of the photoactivity. (Activity was not lost even if the second MeCN run succeeded examination in aqueous KI solution, for which no photoresponse was detected.)

Figure 1 shows the current–potential curve of the film-coated RDE irradiated with a chopped Ar laser beam in the iodide-containing electrolyte. The photocurrent is shown both as a band superimposed on the dark current and as the filtered and rectified modulated component. The figure insert shows slow (0.2 Hz) chop experiments amplified in the region of highest photoactivity. We find that cathodic currents due to the reduction of the film, or anodic currents for its irreversible oxidation at much more positive potentials in non-iodide-containing $(TBA)ClO_4$,[4] have no, or barely detectable, photoresponse.

Since the film is partly transparent, it cannot be excluded, a priori, that the observed photoeffects do not result from a Schottky barrier at the metal–film interface. However, such a barrier is unlikely because the photoeffect should then modulate all solution currents as a change in series voltage, which is not observed. We have made junctions with Pt, Au, and Pd electrodes, which cover a spread in work function of about 0.5 V, without seeing different behavior than that described here for Pt electrodes. Typically, barriers to p-type films are made with metals of lower work function as well. Thus the data contradict a buried p-type junction as source of the photovoltaic response.

Photovoltage and photocurrent as a function of input intensity are shown in Figure 2a,b, respectively. The irradiance of the RDE was measured by a radiometric silicon detector placed in the same geometry. A negative shift of electrode potential of 74 mV/decade laser input intensity is seen from Figure 2a, translating to a quality factor of 1.25 for the photodiode. The photocurrent at a potential of 0.260 V vs SCE (positive shift from Figure 1 because of lower iodide concentration) is initially linear with intensity, less so at higher intensity. This may be partly due to the lack of a distinct photocurrent plateau as a function of potential and the low concentration of iodide. The high dark current at positive potentials indicates that the cast films are porous, a further complication.

The excitation spectrum of the photocurrent, measured with 14-Hz chopped light under constant potential, is given in Figure 3, corrected for monochromator–light source throughput with a ±5% flat detector. The RDE was fixed in a horizontal mount next to an optical flat window in this experiment. The cell was completed with a Pt-ring counter electrode and Ag quasi-reference. It should be noted that absorbance of C_{60} solutions in hexanes extends to above 620 nm[1,3,10] and that absorption spectra of sublimed films of C_{60} have a small, broad peak around 640 nm.[11] The spectrum of the non-solvent-containing, sublimed film and the photoexcitation spectrum are generally similar. Photoactivity is detectable to around 720–740 nm, corresponding to about a 1.7-eV band gap. A previous measurement of the band gap from photoemission of C_{60}^- ions also yielded 1.7 eV.[12]

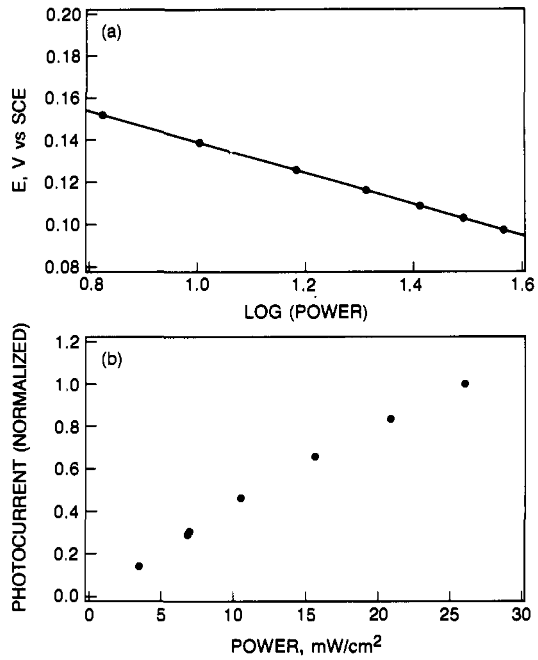

Figure 2. (a) Open circuit potential of C_{60}-filmed Pt RDE under cw Ar laser illumination. [(TBA)I] = 1 mM. (b) Photocurrent at RDE of part (a) measured at 0.260 V vs SCE under 14-Hz chopped Ar laser illumination, as in Figure 1 (dashed line) except [(TBA)I] = 1 mM.

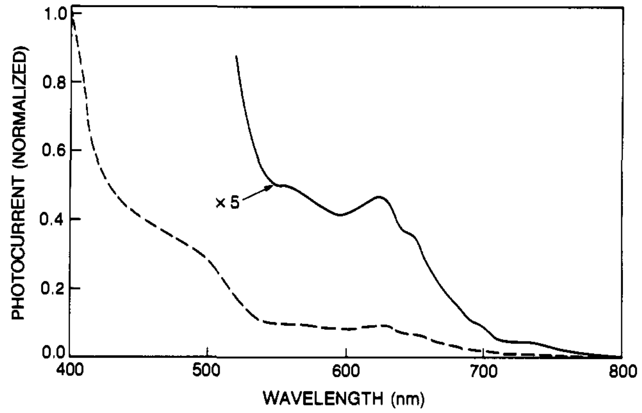

Figure 3. Photoexcitation spectrum measured at 0.02 V vs SCE (actual reference for noise reduction was a Ag wire) by using 14-Hz chopped monochromatic irradiance and a 7–28-Hz band-pass filter at 100× gain (Rockland 1022F) as preamp to a Model 128 PAR lock-in amplifier. The light source was a 100-W halogen bulb, and wavelengths were measured with an Oriel 7240 monochromator (1-mm slits). The RDE was fixed horizontally with a concentric Pt-ring counter electrode in 0.1 M (TBA)I.

Electrochemical evidence for iodide oxidation as a result of the photocurrent was obtained by repeating the experiment of the insert in Figure 1 with a rotating ring–disk electrode (RRDE) configuration. The C_{60} film was cast onto the disk, but not the ring, taking advantage of the low surface energy of the fluorocarbon spacer between the concentric electrodes over which the benzene does not spread. For chopping frequencies low with respect to the RRDE transit time, the cathodic ring current response, at potentials (−0.4 V vs SCE) negative enough for the

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limiting current collection of iodine, mimics the anodic photocurrent, with ratio corresponding to that found for the collection of these species in the dark current on the iodide wave.

These results demonstrate that C_{60} films have distinct n-type semiconductor character. Although the photovoltage and quantum efficiency are small at this stage, they have been studied only under a limited range of conditions. The effects measured here are consistent with photovoltaic junction response, but may also be modified by the photoconductive contribution.[5] We have previously noted such combined effects in junctions of a-Si:H.[13]

Work is ongoing to clarify solvent and ion effects, the extension to C_{70}, and the comparison to behavior of sublimed C_{60} films. For the latter two cases, preliminary experiments indicate photoanodic behavior at positive potentials paralleling cast C_{60} for both, while the sublimed film shows photocapacitive response at potentials negative of open circuit. We note that, although C_{60} is now known to be photodegradable[14] by UV light, no loss of photoactivity was detected in successive repetitions of voltammetry under the Ar ion laser (broadband) short term exposure in these experiments.

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Regeneration and Reduction of Native Bovine Pancreatic Ribonuclease A with Oxidized and Reduced Dithiothreitol

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We report here the first successful regeneration of bovine pancreatic ribonuclease (RNase A) with the cyclic disulfide *trans*-4,5-dihydroxy-1,2-dithiane (DTT^{ox}) under anaerobic conditions. We also report the isolation of a native-like three-disulfide species lacking the 65–72 disulfide upon reduction of native RNase A with dithiothreitol (DTT^{red}). These results represent a significant experimental breakthrough in attempts to elucidate the regeneration pathways of RNase A and clarify a considerable amount of controversy that has arisen in the literature. The experimental techniques used to obtain these results offer the potential to resolve similar problems in other proteins and will, along with the bulk of the experimental data, be presented in greater detail elsewhere.

RNase A has been regenerated successfully with mixtures of oxidized glutathione (GSSG) and reduced glutathione (GSH),[1-4] and a number of regeneration pathways have been assigned on the basis of a kinetic analysis.[5] However, the determination of the specific disulfide-bonded intermediates that are involved in these pathways has been hampered by the complexity of the problem. Linear disulfide reagents, such as GSSG, can form stable mixed disulfides with protein thiols.[4,6] This increases the number of possible disulfide-bonded species from 763 to 7192.[7] In addition, the stability of various mixed disulfides with GSSG strongly determines which pathway is taken.[5] Because mixed disulfides with DTT^{ox} are highly unstable,[6] these problems do not exist when DTT^{ox} is used.

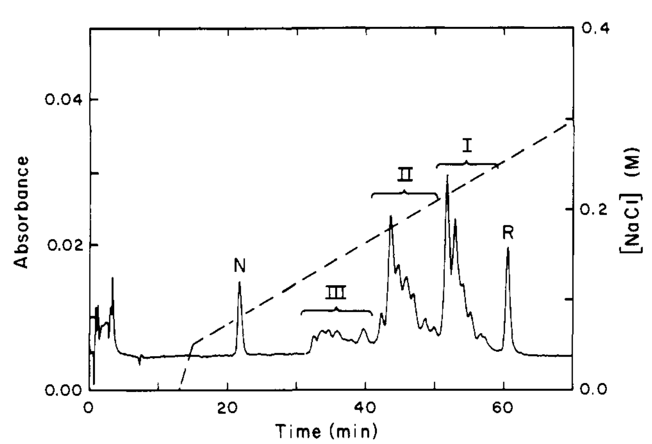

Figure 1. The solid curve represents the chromatographic separation of disulfide intermediates in the regeneration of reduced RNase A on a Bakerbond CBX column at room temperature in 25 mM Hepes, 1 mM EDTA, pH 7.0. Fully reduced RNase A (30 μM) was regenerated for 90 min at 25 °C in 100 mM Tris, 2 mM EDTA, pH 8.0. The starting concentration of DTT^{ox} was 100 mM. The regeneration process was quenched by addition of an excess of AEMTS and desalted. R is the fully reduced protein, N is the fully regenerated native protein. The Roman numerals indicate the number of intramolecular disulfide bonds of the protein in the labeled peak. The peaks at the beginning of the chromatogram pertain to buffer salts. The salt gradient used to elute the protein is represented by the dashed line.

Previously published attempts to use DTT^{ox} in studies of the regeneration pathways of RNase A have been unsuccessful, because of an apparent inability of DTT^{ox} to regenerate RNase A.[3,8-10] A report by Pigiet and Schuster[11] indicating that DTT^{ox} can regenerate RNase A is complicated by the failure to exclude oxygen; hence, the relative contributions of air oxidation and DTT^{ox} to the regeneration process could not be evaluated.[12,13] Since DTT^{red} is a potent reducing agent,[14] the amount of DTT^{red} produced by the formation of protein disulfides inhibits the rate of regeneration. Since the thiolate anion represents the relevant reduced species responsible for the reducing power[6] of DTT^{red}, a decrease in pH will increase the oxidative strength of the redox pair significantly. While previous studies[3,8-10] have been conducted at pH 8.7, the majority of our experiments have been carried out at pH 8.0, the pH used in the previous glutathione studies of Konishi et al.[4,5] Typical results are shown in Figure 1.

An additional factor in the failure of previous workers to detect native RNase A is the lag time between the start of the oxidation process and the appearance of the native protein. This lag varies from 15–90 min and depends on the pH, temperature, and starting concentrations of DTT^{ox}, DTT^{red}, and protein. In previous regeneration studies[3,8,10] data were taken only during the first 30 min of the oxidative process. We have observed that, even under the less favorable conditions used in those earlier studies,[3,15] an apparent half-life following the lag period $(\tau_{1/2})$[16] of 34 h can be

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