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Chemistry

Picosecond dynamics of iron proteins

(picosecond spectroscopy/cytochromes/porphyrins)

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ABSTRACT **The excitation of hemoproteins containing either Fe^{2+} or Fe^{3+} with a picosecond light pulse resulted in the bleaching and recovery of the iron absorbance bands within 6 psec. A new absorbance band also was observed whose formation and decay rate of less than 6 psec suggests that it is probably due to $S_1 \rightarrow S_n$ absorbance. The Soret band relaxation time is deduced to be ~0.5 psec whereas the free base hemoprotein does not show any of the above fast-decay characteristics. Therefore, there is a strong influence of the metal on the energy dissipation process.**

The relaxation rates of proteins and the role of their structure on the energy dissipation mechanism has been the subject of a considerable research effort (1–4). The dynamics of electronically excited states of hemoproteins and iron-containing porphyrins have been measured mainly by studying their fluorescence properties. In the case in which the relaxation is mostly via nonradiative channels, the electronic relaxation processes are studied by other methods, such as resonance Raman spectroscopy and resonance fluorescence of hemoproteins and metalloporphyrins (5–7), which have provided critical insight into the relaxation mechanism of these processes.

Adar *et al.* (5) have measured the resonance Raman spectra of five hemoproteins and, from the relationship of the bandwidth and time, they calculated relaxation lifetimes of corresponding excited states. It seems, though, that in some cases, relaxation times calculated from uncertainty principle considerations would be upper limits because the bands quite possibly could be heterogeneously broadened. In the case of cytochrome *c*, for which a considerable amount of information exists, one finds that the resonance Raman spectrum is quite similar to that of the bipyridine complex of its isolated heme, iron protoporphyrin IX (8). Its low-temperature absorbance spectrum also reveals a fine structure that is associated with the α and β bands of the native cytochrome *c*. This fine structure is an integral part of the protein integrity and is not observed when the protein is denatured at alkaline pH or digested with pepsin.

With the development of picosecond spectroscopy (9) to its present capability of simultaneous wavelength and time resolution (10, 11), one can measure directly the role of the metal and protein in the energy dissipation mechanism of hemoproteins. Aspects of the important catalytic activity, oxygen binding properties, and involvement in energy transduction processes by the hemoproteins can also be studied by this method. The suggestions that vibrational energy transfer within the protein skeleton may be influential in oxidation and photophosphorylation (12, 13) make it desirable to study the excited-state lifetimes of hemes and hemoproteins, especially with respect to the role played by the protein and the metal in excited-state relaxation. The experiments and data presented here attempt to elucidate these processes in some iron-containing proteins and to correlate them with the studies by Magde *et al.* (14) on the relaxation of metal porphyrins

MATERIALS AND METHODS

Cytochrome *c* (horse heart type VI), cytochrome *c* peptide (Microperoxidase) obtained on peptic digestion of cytochrome *c* and containing amino acid sequence 11–21, hematoporphyrin, ferriprotoporphyrin IX chloride (hemin), ferriprotoporphyrin IX hydroxide (hematin), and horse heart myoglobin were purchased from Sigma Chemical Co. and used without further purification. All other chemicals were reagent grade. Experiments were performed at room temperature in pyridine/10 mM phosphate buffer (20:80, vol/vol), pH 7.0–7.4.

The absorbance of the solution was adjusted to 0.5–0.7 at 530 nm in a 2-mm optical path length cell. Reduction from the ferric to the ferrous state was accomplished by adding a minimal amount of solid $Na_2S_2O_4$ to the cuvettes. We verified the state of oxidation or reduction by the absorbance spectra.

Two optical experimental systems were used in this study. Each one provided an intense single picosecond pulse of sample excitation and a relatively low-intensity pulse train, for interrogation, that on its own did not induce any observable absorbance changes.

In all experiments described, we used a passively modelocked Nd^{3+} glass laser. Fig. 1 *upper* shows the optical arrangement (15). The laser generates the ~6-psec excitation pulse (530 nm, ~10 mJ) and broad band picosecond continuum that, after passing through an echelon, is used as the interrogating light source in the wavelength range 480–800 nm. The second experimental setup utilized a 6-psec 355-nm pulse for sample excitation and a picosecond dye laser (to be described elsewhere) for sample interrogation in the wavelength range 400–480 nm.

The echelon transforms the continuum into seven pulses with an interpulse separation of either 20 psec or 6.7 psec. This echelon-induced train is then split into two sets of seven segments each by a pellicle beam splitter (no. 10 in Fig. 1 *upper*); these sets form the reference I_0 and interrogating I beams of the double-beam picosecond spectrometer.

Two distinct experimental configurations were used. In the first case, the time sequence was as follows: the reference echelon train I_0 traversed the protein cell 300 psec before the excitation pulse. The second or third pulse of the probing echelon train I intersected the excitation pulse in the protein cell. Thus, the first sequence of pulses (I_0) probed the molecules to be excited 300 psec before excitation, providing a true reference calibration, whereas the I sequence monitored changes during and after excitation. Such a procedure not only enabled us to measure ΔA and eliminate ambiguities which arise from

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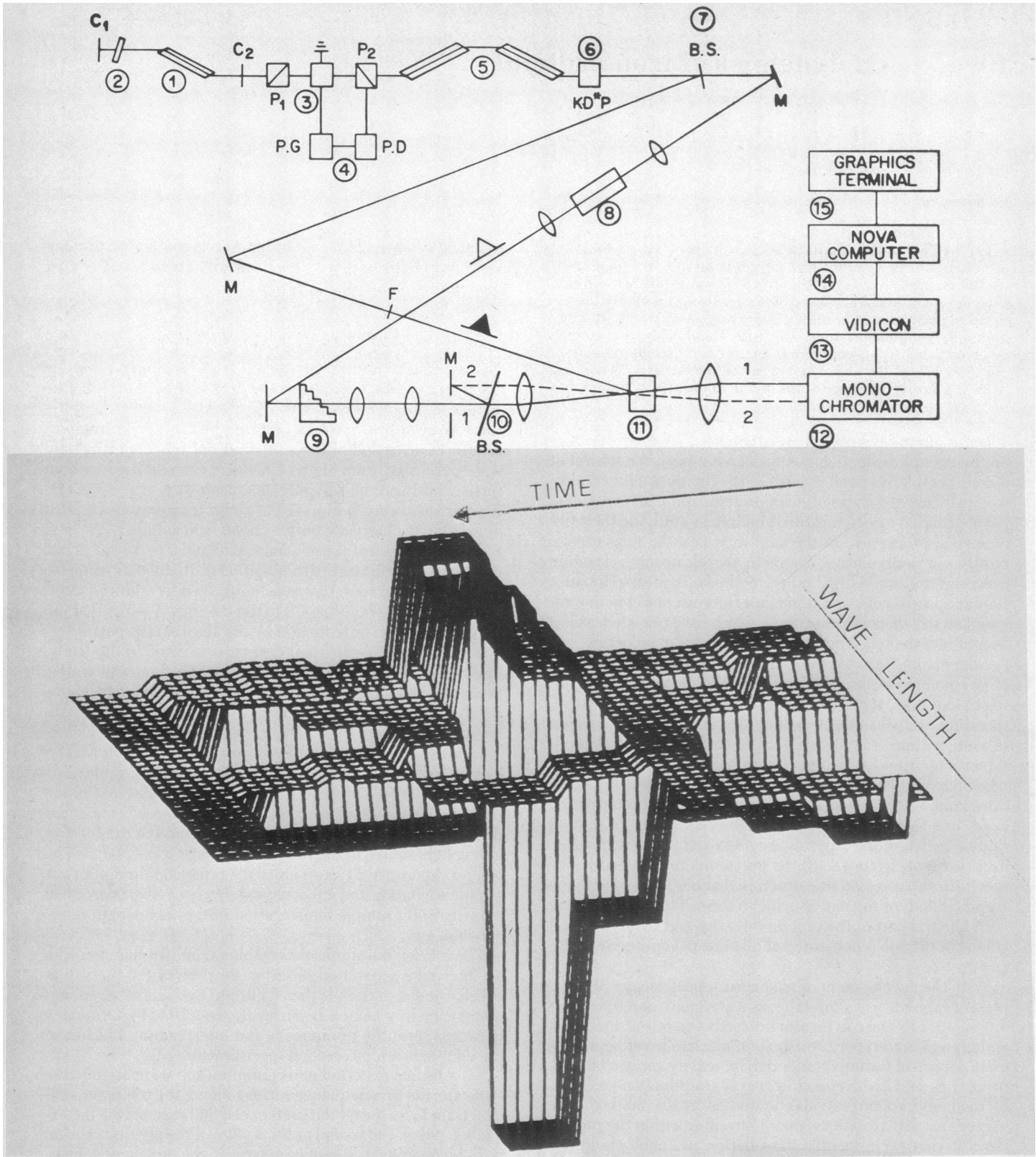

FIG. 1. (*Upper*). Schematic representation of the experimental system. The components are (*1*) Nd^{3+} glass oscillator with cavity mirrors C_1 and C_2, (*2*) saturable dye absorber cell, (*3*) Pockels cell positioned between crossed polarizers P_1 and P_2, (*4*) electronic Pockels cell driver, (*5*) Nd^{3+} glass amplifiers, (*6*) second harmonic generator, (*7*) beam splitter, (*8*) broad band continuum cell containing CCl_4 or cyclohexane, (*9*) variable delay transmission echelon, (*10*) double-beam splitter, (*11*) sample cell, (*12*) monochromator, (*13*) optical data digitizer, (*14*) Nova computer, and (*15*) graphics terminal. Mirrors are denoted by M; optical filters by F. ▲, 530 nm (excitation); △, 560–1600 nm continuum (interrogating). (*Lower*). Simultaneous three-dimensional display of time-dependent spectrum of reduced cytochrome *c* excited by a single 6-psec 530-nm pulse and interrogated in the wavelength region 550–570 nm. The time element was provided by a stepped delay echelon and the spectral resolution was provided by a spectrograph. Note that the 550-nm band is bleached, and in the 560–570 nm range, a new absorbance developed simultaneously. The recovery of the 550-nm band and decay of the 560–570 nm band was found to take place within 12 psec.

shot-to-shot variation but also corrected for possible sample photodegradation. In the second case, a segment of the first beam entered the sample simultaneously with the excitation whereas the rest of this beam and all of the second one entered the cell subsequently. The first echelon train, which is labeled as beam no. 1 in Fig. 1 *upper*, interrogated the time domain −10 to 100 psec after excitation whereas the second set (indicated as beam no. 2) probed the time period 400–500 psec after excitation.

For all experiments, the echelon segments were sharply imaged onto distinct spatial regions of a N_2-cooled RCA 4532 silicon vidicon (9). The vidicon scan was controlled over a programmable two-dimensional grid consisting of 512 × 512 resolvable points. The horizontal vidicon axis (x axis) provided the wavelength coordinate; the vertical axis (along the spectrometer entrance slit y axis) formed the time element. The total horizontal vidicon scanning length that corresponded to the overall wavelength width was adjusted to be between 125 and 350 Å. The two-dimensional grid scanning procedure split the rectangular grid into five or eight equal rectangular portions, each one corresponding to a preselected but different center wavelength, each having the same spatial configuration. This arrangement was used for the display of data in a three-dimensional plot, as shown in Fig. 1 *lower*, in the form of time in psec (x axis) versus wavelength in nm (y axis) versus intensity (z axis). When data for a particular wavelength were sufficient, a one-dimensional plot of the echelon segments versus intensity was plotted by the computer on the graphic terminal and copied by a hard copy unit, resulting in a graph similar to the one given in Fig. 3 where the absorbance changes are plotted as a function of time. In this case, as in all the data presented, each segment corresponded to a specific pulse in the interrogating train.

For greater accuracy, we also alternated between measuring the changes in the sample with and without excitation while the probe echelon sequence (interrogating pulses) traveled through the sample with each shot. ΔA was calculated for each time (echelon) segment by evaluating $\Delta A = \log (I^w/I^n)$, in which I^w and I^n refer to the intensity of the interrogating pulse in the presence and in the absence of the excitation pulse, respectively. The effect of excitation intensity and reliability of the data becomes more evident by considering that in most experiments discussed the ratio of ΔA with and without excitation is ~10—i.e., $\Delta A_{(with)} = 0.3$ and $\Delta A_{(without)} = 0.03$.

RESULTS

By means of the picosecond apparatus we measured ΔA in aqueous solution as a function of time and wavelength after excitation, with a single 6-psec, 530- or 355-nm pulse, of (*i*) hematoporphyrin, (*ii*) bipyridine heme, (*iii*) cytochrome *c* peptide, (*iv*) cytochrome *c*, and (*v*) myoglobin (excited only with a 530-nm pulse). The results are divided into three categories: reduced-state compounds with the iron in the Fe^{2+} state; compounds with the metal in the Fe^{3+} state; and iron-free proteins.

Fe^{2+} Compounds (Reduced Heme). The absorption spectrum of cytochrome *c* containing Fe^{2+} (Fig. 2) consisted of two relatively narrow bands at 550 nm [Q(1, 0) (α band)] and 520 nm [Q(0, 1) (β band)] associated with the Fe^{2+} porphyrin group in addition to the Soret band at 420 nm. Excitation of a 0.6-*A* sample with 530-nm light resulted in the immediate bleaching of both 520- and 550-nm bands associated with Fe^{2+}. Within <6 psec, the bleached bands had recovered to their original intensity maximum (Fig. 3 *upper*).

The effect of the 530-nm excitation was investigated throughout the visible and near infrared region by the use of the picosecond continuum. We found that a new wide absorbance band was formed simultaneously with the diminution of the two narrow α and β bands. The kinetics of formation and decay of this new band were monitored as 480–500 nm and 560–750 nm, respectively. Fig. 3 *lower* shows the kinetics at 590 nm. Within the time period of excitation pulse, the band at 500 nm achieved its maximum intensity and decayed to its preexcitation form. It should be noted that no structure was observed in this short-lived absorbance band; in the 880-nm region, we did not observe any absorbance band changes whereas at 750 nm we found changes similar to those at 650 nm.

We were not able to investigate changes occurring after excitation at 355 nm because the reducing agent $Na_2S_2O_4$ absorbed at this wavelength. Results similar to the ones shown for cytochrome *c* were obtained with cytochrome *c* peptide, myoglobin, and bipyridine heme.

Fe^{3+} (Oxidized Heme). In their Fe^{3+} oxidized state, the hemoproteins exhibit a single broad absorbance band at 530 nm. The absorbance cross section of this band assigned to the oxidized metal complex was less, by a factor of 4, than that for the Fe^{2+} band at 550 nm. Within 6 psec after excitation of the Fe^{3+} compounds, $A_{530} = 0.6$, and the intensity of the 530-nm absorbance band decreased and recovered to its original intensity. The ΔA observed for Fe^{3+} compounds at 530 nm was 0.1 compared to 0.5 for the reduced compounds at 550 nm. As in the case of the reduced species, excitation of the oxidized state resulted in the formation of a new absorbance band in the region 480–750 nm with a rate faster than 1.3×10^{11} sec^{-1}. By monitoring the ΔA of these samples at lower wavelengths by means of the tunable picosecond dye laser emission after excitation at 355 nm, we found that the Soret band intensity at 405 nm first decreased and then recovered with a very fast rate. This kinetic behavior of Fe^{3+}–hemin bipyridine is plotted in Fig. 4. Similar behavior was found for all the heme compounds measured. We could not monitor similar changes in the reduced heme because of the absorbance in this region by the $Na_2S_2O_4$ reducing agent.

Iron-Free Proteins. To ascertain the role of the iron, we performed similar experiments with iron-free hematoporphyrins. It can be seen in Fig. 5 *lower* that an instantaneous absorbance was evident but, in contrast to the iron-containing compounds, no decay was observed even after 500 psec. Similarly, the Soret band of the iron-free hematoporphyrins decreased in intensity within 6 psec after excitation with a 355-nm laser pulse; however, no recovery was detected after several hundred psec.

DISCUSSION

The relaxation kinetics of the first excited [Q(0, 0) and Q(0, 1)] electronic states located at 550 and 520 nm, respectively, and the Soret band of metal-free porphyrin heme and hemoproteins are known to be strongly influenced by both metal and protein. Iron porphyrins in the oxidation states of +2 and +3 were excited by means of a single 530- or 355-nm ~6-psec pulse and showed the following behavior. (*i*) Excitation by a 530-nm pulse caused the bleaching and recovery of the 520-nm, 550-nm, and Soret bands; these two events occurred within a 6-psec interval. (*ii*) There was an increase in absorbance in the range 480–750 nm with decay occurring also within the same period of time for both iron oxidation states (Fe^{+2} and Fe^{+3}). (*iii*) The shape of the interrogating pulse segments was symmetrical and remained the same before and after the bleaching or absorption. This lack of distortion of the interrogating pulses may indicate

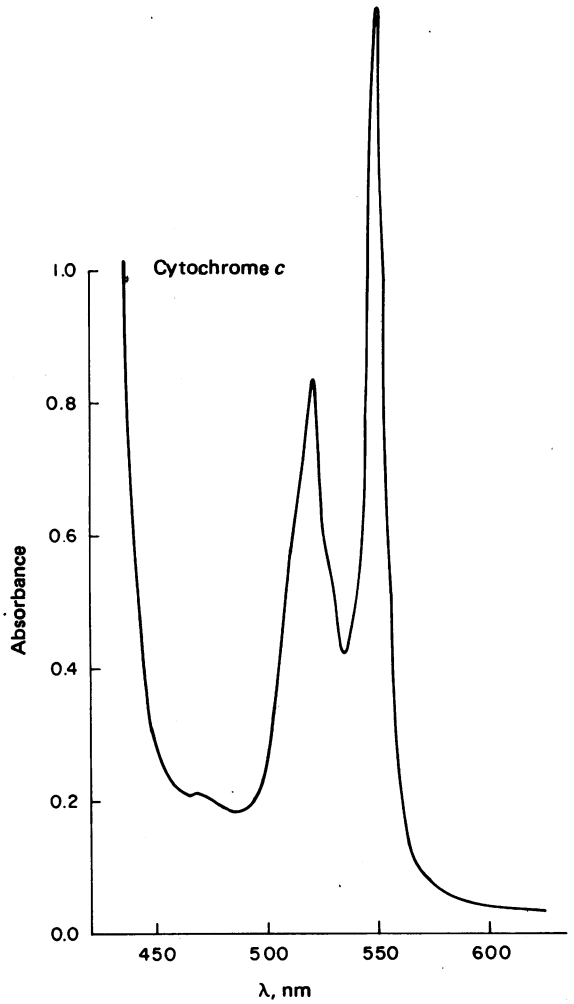

FIG. 2. Absorbance spectra of Fe^{2+} cytochrome *c* in a mixture of pyridine (20%)/10 mM phosphate buffer, pH 7.4 (80%).

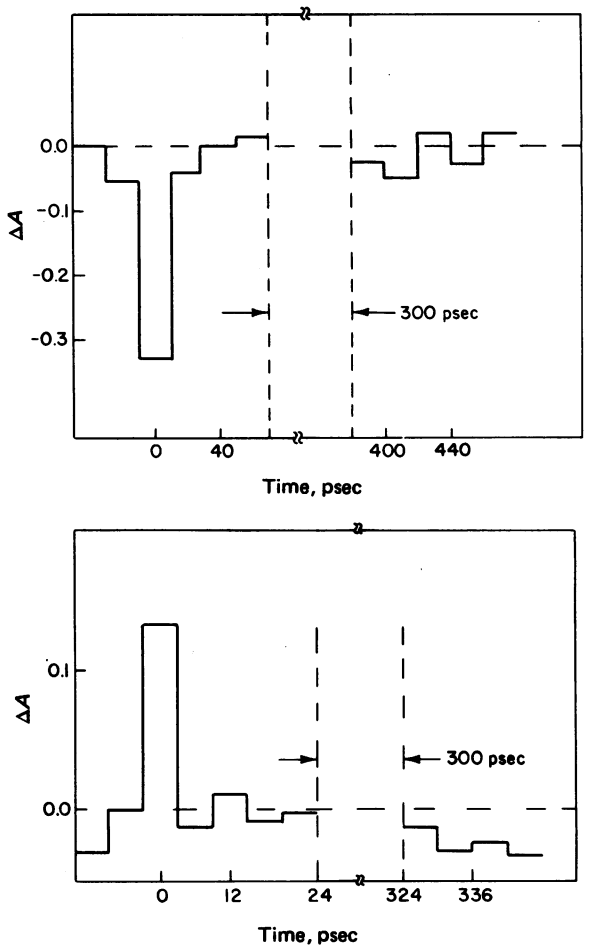

FIG. 3. (*Upper*). Bleaching and recovery kinetics of Fe^{2+} cytochrome *c* monitored at 550 nm by a 20-psec resolution echelon after excitation with a 530-nm, picosecond pulse. (*Lower*). Histogram of the absorbance kinetics at 590 nm in cytochrome *c* heme peptide in the oxidized Fe^{3+} state after excitation with a 530-nm pulse. The time element was a 6.7 psec per segment echelon.

that the formation and decay processes are fast compared to the pulse width. Analysis of our data also suggests that the relaxation time of the protein is faster than the excitation pulse by at least a factor of 3—i.e., less than $\sim 2 \times 10^{-12}$ sec. (*iv*) The bleaching caused by the 530-nm 5-mJ pulse was subsequently monitored at the 550-nm band of the Fe^{+2} state with a 6-psec echelon segment. We observed a 0.5 ± 0.1 *A* bleaching at this wavelength; in addition, there was an increase in absorbance also at 550 nm due to absorption between upper excited states. This becomes obvious by the 0.2-*A* increase at 560 nm where the ground state and upper state absorptions do not overlap. Therefore, the total depletion of the ground state by 530-nm excitation absorbance is equivalent to 0.7 *A*. The ground state repopulation time τ can now be evaluated, assuming steady state, by $\Delta A/A = X/(1 + X)$, in which $X = \tau\sigma lI$ ($\sigma_{550\ \text{maximum}} = 2 \times 10^{-16}\ \text{cm}^{-2}$ and represents the molecular cross section); $l = 0.2$ cm, the optical path length of the cell; and $I = 10^{28}$ photons per sec per cm^2 and represents the photon flux for a 530-nm 5-mJ pulse (which was partially focused into the sample). With these assumptions, we estimate the time for regeneration of the ground state of the Fe^{+2} compounds as ~ 1 psec. (*v*) We applied similar reasoning for calculating the bleaching of the Soret band of the Fe^{+3} compounds after excitation with a single 355-nm pulse. Taking into account that the number of photons contained in the 353-nm pulse is ~ 7.5 times smaller than in the 530-nm pulse and that the noniron hematoporphyrin can be bleached with $\Delta A \sim 1$, we estimate that the Soret band relaxation time will be about 0.5 psec. Although we were prevented by the $Na_2S_2O_4$ absorbance from monitoring the changes in the reduced hemes, we expect that similar kinetics will take place in this region.

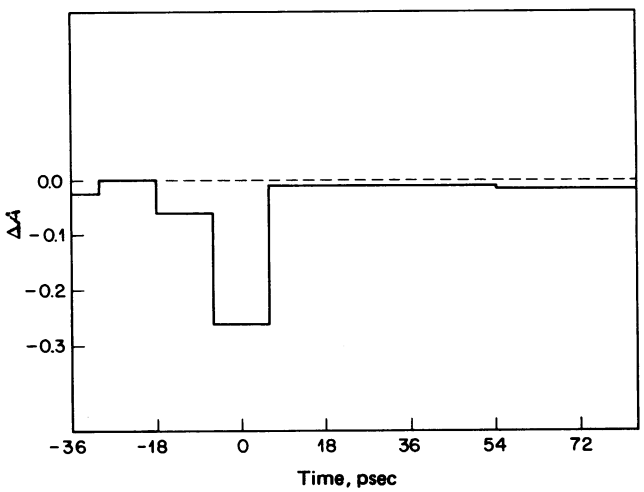

FIG. 4. Bleaching kinetics of hemin bipyridine at 405 nm excited by a 355-nm, picosecond pulse. Similar kinetics were observed with other hemoproteins.

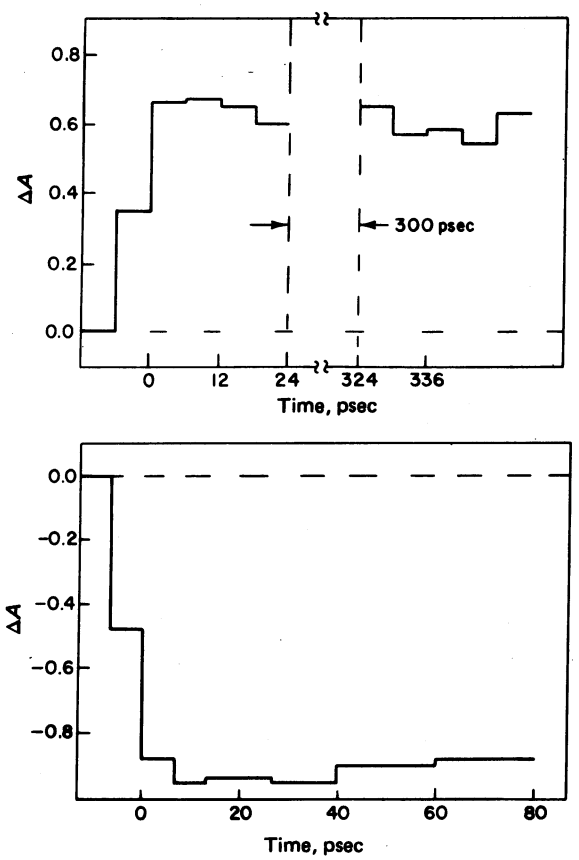

FIG. 5. (*Upper*). Absorbance increase at 590 nm as a function of time observed in hematoporphyrin after excitation with a 530-nm, picosecond pulse. Note that the actual interrogation time was extended to 400 psec. (*Lower*). Bleaching kinetics of hematoporphyrin interrogated in the 405-nm region after being excited by a 355-nm pulse.

The data presented indicate that the electronic relaxation of iron protoporphyrin IX covalently bound to protein (cytochrome *c*) and noncovalently bound to protein (myoglobin) takes place within 1 psec after excitation. Although the formation rate of the new bands in all compounds studied is in the 10^{12} sec^{-1} range, the decay is fast for the iron-containing species (<6 psec) and much slower (≫500 psec) for the iron-free species, such as the bipyridine complex. This finding substantiates the proposal that the relaxation rate of iron proteins is fast regardless of the state of oxidation of the iron.

Of course, the very long lifetime of the excited state of iron-free hematoporphyrin IX is expected because these compounds are known to exhibit strong fluorescence and relatively long lifetimes. The ultrafast relaxation of the iron-containing proteins raises difficulties in determining the influence if any of the protein on the decay rate of the electronic excitation. Within experimental error, the Soret band and the α and β absorbance Fe^{2+} bands relax with the same rate to the ground state regardless of whether they are excited with 530- or 353-nm light. This statement does not imply that the relaxation rate is equal in both bands but rather that both are faster than 6×10^{-12} sec. Such very fast relaxation processes have been observed in even simpler systems such as azulene in which the relaxation rate was measured to be about 7 psec (9, 16), 4 ± 4 psec (17, 18), or 2.5 psec (19).

The increase in absorbance between 480 and 750 nm (with the exception of narrow bleaching bands at 520 and 550 nm) is assumed to be due to higher state absorption between excited electronic states. If this assumption is correct, the lifetime of the excited state determined by observing the bleaching should be the same as that determined by observing the absorbance increase. The experiments presented in this paper verify this assumption and a lifetime of <3 psec for the Q band of the iron hemeprotein is dictated by our results.

The nature and the influence of the protein on the relaxation of the electronically excited state of this heme cannot be ascertained yet. We expect though that by the use of other metal porphyrins with longer relaxation lifetimes, one may be able to diagnose the role of the protein and metal on the energy dissipation processes.

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