QUANTITATIVE RADIATION EFFECTS IN HEMOPROTEINS AND THEIR CONSTITUENTS

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ABSTRACT In order to verify the "radiation protection" effect of prosthetic groups in irradiated conjugated proteins and the influence of their electronic structure on intramolecular excitation transfer, two series of substances were examined. The first was composed of ferrihemoglobin and its constituents and the second was composed of similar hemoproteins with heme groups with major differences in electronic structure. The results point to a strong radiation protection effect of the heme groups and a dependence of radical yield of hemoproteins on the electronic structure of the heme. A possible interpretation of this latter effect is discussed.

INTRODUCTION

In recent years much attention has been devoted to the quantitative aspects of radiation effects in biological and biologically interesting molecules in the dry state. Though it is evident that these systems are oversimplified models of the biological processes in which these molecules are involved in vivo, this approach is meaningfully related to the general problem of the structure-function relation in biological molecules. In fact, this is a good, direct method to obtain quantitative measurements of the effects of the molecular structure on energy absorption and transfer. Consequently, a probing of the physical mechanisms of these phenomena may make easier the understanding of the biological processes in which intermolecular exchange of energy is involved.

On this basis, in particular, the radiation resistance and the radical yield of several proteins irradiated with X- or γ-rays have been measured, and the role played by aromatic and sulphur-containing amino acids in the radical formation processes has been carefully examined (1, 2). However, the problem of radiation effects in conjugated proteins has not yet been given the attention it deserves, considering the highly important biological function of these molecules. In fact, only the ceruloplasmin (3) and the nucleohistone (4) have been studied.

The results obtained in these cases suggest the hypothesis that the conjugated group might play the role of a sink of absorbed energy, in the sense that the proteins, as a consequence of their volume and radiation sensitivity, could be the principal

target of the radiation, and the prosthetic group could essentially dissipate the excitation energy absorbed by the conjugated molecule. In this sense we will speak of a radiation protection effect of the conjugated group to the heme-free protein. Checcucci and Crippa (5) gave evidence in a previous paper of such a radiation protective effect of the prosthetic group on hemoproteins. With this hypothesis there still remains an open problem concerning the mechanism of excitation transfer from the protein to the conjugated group. In fact, the radiation damage observed in conjugated proteins is identical in any case and is identifiable with the peptide free radical described by Gordy and Shields (6). The number of free radicals, G, induced in identical experimental conditions by 100 ev absorbed varies, however, in similar molecules characterized by a different linkage between the prosthetic group and the protein chains (7). This fact led us to suppose that the intramolecular excitation transfer is related to the linkage between the prosthetic group and the protein, but this is not sufficient to explain the effect of radiation protection by the prosthetic group. In other words, at this point the connection between radiation resistance and structural properties of this type of molecule does not seem to us to be completely clear. We have supposed, therefore, that the electronic structure of the heme linked to the protein may play a prominent role in the localization of the excitation energy; the theory of Duchesne (8) and Pullman (9) on this subject, in which the increase in radiation resistance as a function of the degree of delocalization of π-electrons is considered, is suitable. This theory has been experimentally confirmed in simple molecules by several authors (10, 11), but at the present time there is no direct evidence that it is possible to apply this view to the conjugated proteins as well.

Therefore, we have examined the radiation effects on two series of biomolecules and their constituents; the first series, in order to confirm the radiation protective effect of the heme in hemoproteins, was made up of hemiglobin (methemoglobin = ferri-Hb) and its molecular constituents. Our confidence about the possibility of extending the results obtained in the components of hemiglobin to all hemoproteins lies in the general similarity in amino acid composition of these molecules and the deep difference between the values of G of the hemoproteins and of the simple (nonconjugated) proteins.

The second series was composed of similar hemoproteins characterized by heme groups with major differences in electronic structure. A series with this character was used in order to verify the hypothesis that the dissipative properties of excitation energy is related to the electronic structure of the heme.

MATERIALS AND METHODS

Materials

The materials used in experiments were all obtained from the market, except the ferrocytochrome *c* which was prepared from ferricytochrome *c* by reduction in water solution with sodium dithionite (5% w/w) and recrystallization. Globin was obtained from K & K Labs., Inc., Plainview, N.Y. Protoporphyrin IX, hemin, and horse hemiglobin (ferri-Hb) were ob-

tained from Calbiochem AG, Luzerne, Switzerland; horse skeletal-muscle myoglobin and horse hemoglobin (ferro-Hb) were obtained fromc Shuchardt, GmbH, München, Germany and horse-heart ferricytochrome *c* was obtained from Boeringer and Soehne GmbH, Mannheim, Germany.[1]

The oxidation state and ligand bonding on the iron were checked by visible and ultraviolet absorption spectrophotometry in solution (Cary 15 double beam spectrophotometer [Cary, Monrovia, Calif.]) before and after irradiation.

All the substances were used in crystallized state.

Experimental Procedure

All the samples (60–100 mg) were put under vacuum for 30 hr and sealed off at a pressure of 10^{-4}–10^{-5} mm Hg in quartz sample tubes. Each sample was irradiated at room temperature with X-rays (50 kv, 30 ma) at increasing radiation doses. The doses were measured by a PTW Simplex-Universal Dosimeter (Physikalisch-Technische Werkstätten, Freiburg, Germany) equipped with a soft radiation ionization chamber, and the absorbed energy was calculated; it was kept in mind that, at radiation energy lower than 50 kev, the photoelectric absorption is practically the only absorption phenomenon. The data for computation of photoelectric cross-section and details of calculation are reported by Lea (13).

Electron Paramagnetic Resonance (EPR) Spectrometry

The number of induced free radicals was determined, after each irradiation, by analytical measurements of the electron paramagnetic resonance (EPR) recorded spectra (representing the first derivative of the absorption curve), and by comparison with a standard lignite BM3 ($2.0 \pm 0.2 \times 10^{17}$ spins/g) sample measured under the same conditions in a dual sample cavity. A Varian EPR spectrometer (Varian, Palo Alto, Calif.) operating in X band with a 100 kc/sec field modulation was used. All the spectra were recorded at room temperature, and at this temperature, we could ascertain that the radicals were completely stable.

Globin, protoporphyrin IX, myoglobin, and hemiglobin showed an EPR spectrum before irradiation; in these cases the number of radicals was determined by subtraction.

The absolute *G* values can be affected by considerable errors (2), but the reproducibility of data was better than 10%.

Magnetic Susceptibility Measurement

In order to determine the actual spin state of ferrimyoglobin, ferri-Hb, and ferro-Hb, in which a thermal mixing of different spin states is present (14), we performed magnetic susceptibility measurements with a Gouy balance (Newport Instr. Ltd., Newport Pagnell, England).

RESULTS

Table I shows the *G* values obtained for the first series of substances examined. With protoporphyrin IX, the porphyrin ring minus Fe, a *G* value of 0.5 was obtained.

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[1] A remarkable mix-up exists in the nomenclature of these substances. We used the nomenclature adopted in Mahler and Cordes textbook (12) for the hemiglobin (ferri-Hb) constituents globin and hemin = ferriprotoporphyrin IX. The various hemoproteins are indicated in the text with the simplest names adopted in literature.

These results agree with the data previously reported by different authors; in fact the *G* value of the globin is in the range of the values (from 2 to 7) obtained by Henriksen (2) on simple proteins. In the case of the protoporphyrin IX and hemin, no direct comparisons are available; however, one finds basic agreement with the results obtained in similar experimental conditions on phtalocyanines (15).

Figs. 1 *a* and *b* show the number of free radicals induced versus the absorbed radiation doses; the values relative to hemin are not reported because of the enormously low sensitivity of this molecule.

TABLE I

Substances	*G*
Globin	4.3
Ferrihemoglobin	0.4
Ferrohemoglobin	0.8
Hemin	~0.0005

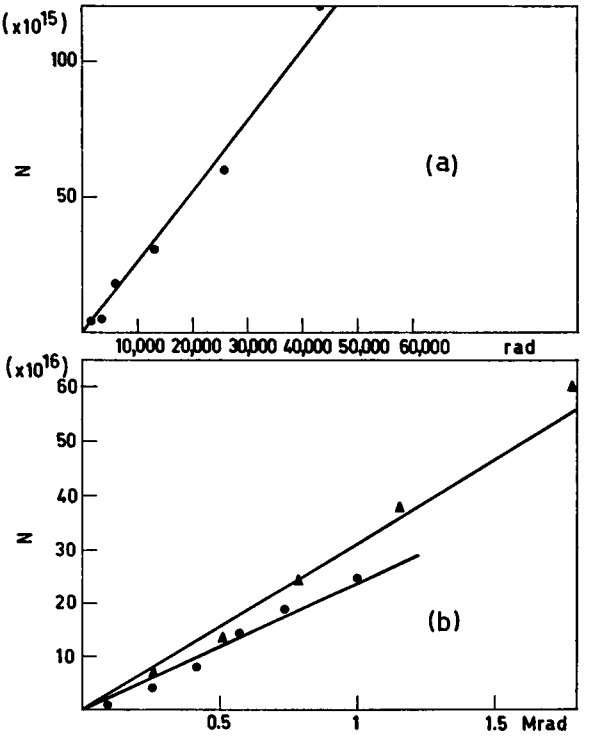

FIGURE 1 (*a*) Radical concentration in globin at room temperature against dose of radiation. (*b*) Radical concentration in protoporphyrin IX (▲) and ferri-Hb (●) at room temperature against dose of radiation.

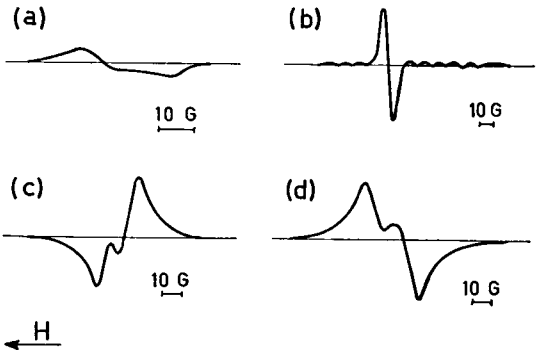

FIGURE 2 EPR spectra of X-irradiated, (*a*) hemin, (*b*) protoporphyrin IX, (*c*) globin, and (*d*) ferri-Hb.

EPR spectra of the various substances are reproduced in Fig. 2. The spectrum of the globin shows the typical peptide doublet of simple proteins (6). The protoporphyrin IX exhibits a very intense and complex spectrum even before X-irradiation; the nature and origin of the radical producing such a spectrum is an unresolved and intriguing problem. The bad resolution of the hemin spectrum is due to the high gain required for the detection of a small number of induced radicals even at enormous doses of absorbed energy.

Table II shows the G values of the series of the hemoproteins examined. The spectrophotometric analysis of these substances made evident that in the case of ferri-myoglobin and ferro-hemoglobin, the group on the Fe sixth ligand position was OH_2. In the case of ferri-hemoglobin, the Fe sixth ligand position was occupied by OH_2 and OH^- groups in a ratio of about 2:1.

TABLE II

Substances	G	π-electrons
Ferrimyoglobin	1.1	26.9
Ferrohemoglobin	0.8	27.0
Ferrihemoglobin	0.4	28.3
Ferricytochrome *c*	0.2	29
Ferrocytochrome *c*	0.1	30

Although the substances studied are very similar in their molecular and physicochemical properties, the values of G exhibit a remarkably progressive decrease. In the following part of this paper, a possible interpretation of this fact will be discussed. Fig. 3 shows the dose-effect plots for these molecules, and in Fig. 4 typical EPR spectra are reproduced.

From the results of the measurements in magnetic susceptibility, χ, the values of magnetic moments in terms of Bohr magnetons were calculated for ferri-myoglobin, ferro-Hb and ferri-Hb by the relation

$$\chi = \chi_{\text{dia}} + N\frac{n^2\mu_B{}^2}{3kT};$$

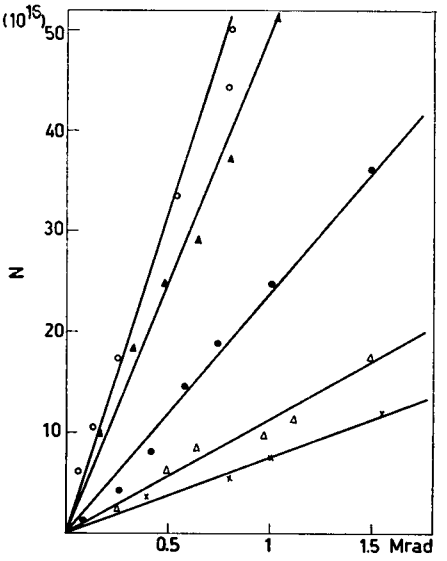

FIGURE 3 Radical concentration against dose of radiation in ferri-Mb (○), ferro-Hb (▲), ferri-Hb (●), ferri-cyt *c* (△), and ferro-cyt *c* (×).

where N is the number of magnetic ions in the sample, n is the Bohr magneton number, k is the Boltzman constant, and T is the absolute temperature.

The values of magnetic moments obtained were, respectively, 4.6 μ_B for ferri-myoglobin, 4.0 μ_B for ferro-Hb, and 2.8 μ_B for ferri-Hb. These values are somewhat smaller than those usually reported by others; this fact can be explained if, following George, Beetlestone, and Griffith (14) and Kotani (16), we keep in mind that the hemes of these substances are thermal mixtures of hemes in high-spin states and low-spin states. As a consequence, the actual magnetic moment is intermediate between the values of the two states. The percentage of hemes in both states cannot be measured a priori because different ligands are present in different amounts. Therefore, the values of the magnetic moments must be determined experimentally for each

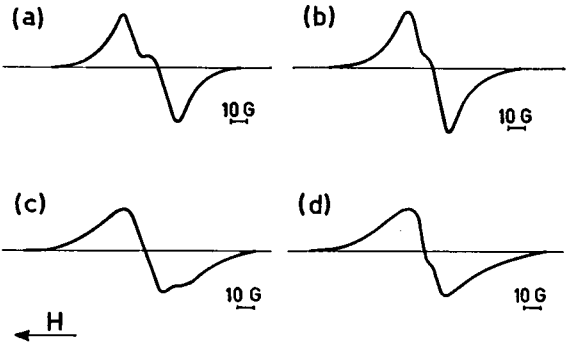

FIGURE 4 EPR spectra of X-irradiated hemoproteins: (*a*) ferro-Hb; (*b*) ferri-Mb; (*c*) ferri-cyt *c*; (*d*) ferro-cyt *c*.

group of substances. The only requirement is that their experimental values fall within the two theoretical extremes. From the values of magnetic moment, one can calculate the resultant spin quantum numbers by the relation

$$n = 2[S(S + 1)]^{1/2}.$$

At this point it is necessary to draw on these values and available theories for consistent information about the heme electronic structure of the molecules studied. It is well known that calculation of the electronic structure of the iron-porphyrin complexes is a very intriguing problem because the mechanism of complex formation lies, at least in part, in the interaction between the spin state of the iron and the molecular orbitals of the porphyrin.

Pullman, Spanjaard, and Berthier (17) calculated the interaction between the electronic orbitals of the porphyrin ring and those of a metal cation. On the basis of the calculation, Pullman and Pullman (18) affirm that in high-spin, essentially ionic, ferrous or ferric complexes (myoglobin, ferro-Hb and ferri-Hb), 26 π-electrons are involved; in low-spin, essentially covalent, ferrous complexes (ferro-cyt *c*), 30 π-electrons are involved; and in low-spin, essentially covalent ferric complexes (ferrri-cyt *c*), 29 π-electrons are present.

On the other hand, in ionic complexes, taking a covalency percentage into account, the total number of π-electrons, N_π, must be recalculated on the basis of an experimental determination of the real spin state.

The following equation can be used:

$$N_\pi = N_l \frac{S - S_h}{S_l - S_h} + N_h \frac{S_l - S}{S_l - S_h},$$

where N_l and N_h are the theoretical numbers of π-electrons, respectively, in purely low- and high-spin states of the hemes and S_h and S_l are theoretical values of the high- and low-total spin numbers respectively.

From the experimental S values and S_h and S_l values reported by Kotani (16), we have calculated the number of π-electrons reported in the last column of Table II.

DISCUSSION

The results show the highly important energy localization effect (radiation protection, in the sense previously specified) of the heme in irradiated conjugated hemoproteins. The remarkable radiation resistance increase induced by the prosthetic group on the apoprotein is quite evident and, as a result, the G values of the entire conjugated proteins are much lower than those of the nonconjugated ones.

One must emphasize that all the G values obtained in conjugated hemoproteins, though showing a remarkable variation, are much lower than the corresponding values of the nonconjugated proteins.

The comparison of G and N_π values (Table II) also shows that the experimental results are consistent with our expectations concerning the relation between radiation resistance and electronic delocalization of the heme. The approximations, both in experimental results and particularly in calculation of π-electron numbers, must be considered and, as a consequence, severe limitations must be placed upon the inferences of this correlation. In any case we think the results are consistent with the assumption that the excitation localization takes place toward a specific group; further clarification of the role of mechanism of excitation transfer must be made, and we are presently undertaking this study.

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