

4.10.4. Univ. of Paris - Curie Institute - Univ. of the Negev, Beer-Sheva

4.10.4.1. LRET in Lysozymes

- (A) Intramolecular Long Range Electron Transfer Between Tyrosine and Tryptophan in Lysozymes. A Pulse Radiolysis Study (226).

A pulse radiolytic study of six different c-type Lysozymes, from hen egg-white (HEWL), turkey egg-white (TEWL), human milk (HuML), horse milk (HoML), camel stomach (CaSL) and tortoise (TorL) is presented. The azide radical, the dibromide, and dichloride radical anions oxidize one or more tryptophan side chains in the Lysozymes. The indolyl radical produced in each of these second order 1-electron oxidation reactions subsequently oxidizes a tyrosine side chain to the phenoxy radical in an intramolecular reaction with rate constants varying from 130 s^{-1} for HEWL to $5 \times 10^4 \text{ s}^{-1}$ for HuML at pH 7 and room temperature. The observed intramolecular reactions are discussed in terms of a Long Range Electron Transfer between the two amino acids. (with Chantal Houee-Levin and Pierre Jolles).

- (B) Some Aspects of the Chemistry and Biology of the Superoxide Radical anion (227).

There is increasing evidence that the superoxide radical anion is produced in many biological reactions and especially in respiration. Also, there are many indications that the participation of this radical in certain biological reactions can ultimately have deleterious effects on the health and well being of certain individuals. Based on pulse radiolytic method of generating superoxide its physical and chemical properties are described. This review gives the present state of research on the formation and reactivity of the superoxide radical anion in biological systems, the physiological function of superoxide dismutase, as well as several enzymatic reactions for which the participation of the radical has not yet been conclusively established. (with Chantal Houee-Levin).

5. COLLABORATION WITH OTHER LABORATORIES.

Our contributions to the field of "Pulse Radiolysis Application to Biochemical Research" led us to collaborate with several research groups.

In Israel:

- Prof. I. Pecht of the Department of Chemical Immunology, the Weizmann Institute of Science with whom a long and very fruitful collaboration on "Electron Transfer Reaction in Proteins" started in 1969. This collaboration resulted in 20 publications.
- Prof. E Riklis of the Radiobiology Laboratory, Nuclear Research Centre-Negev, on "Radicals Involvement in Radioprotection and Sensitization".
- Dr. I. Rosenthal of the Department of Technology, Volcany Institute of Agricultural Research, on "Studies on DNA Structural Units and Analogues". This collaboration resulted in 6 publications.

International collaborations:

- Prof. G.E. Adams and Dr. J.L. Redpath of the Gray Laboratory, Mount Vernon hospital, Northwood, Middlesex, England in the field of "Pulse Radiolysis Studies in Peptides and Proteins". This collaboration resulted in one publication and two communications
- The Department of Chemistry, Ohio State University, Columbus, Ohio, U.S.A. This collaboration, first with Prof. L.M. Dorfman and later with Prof. M.H. Klapper dealt with "Pulse Radiolysis Application to Protein Chemistry". It started in 1976 and continues to date. Prof. Klapper spent three Sabbatical with us in Israel (1978/79, 1981/82 and 1987). This collaboration resulted in 16 publications and four awarded grant proposals.
- The department of Nuclear Medicine and Radiobiology, University of Sherbrooke, Sherbrooke Canada. This collaboration started in 1985, first with Prof. J.P. Jay-Gerin on "Biopolymers as Semiconductors. A Theoretical Study". This collaboration resulted with two publications. Since 1990 I have started a collaboration with Dr. Houde on "Long Range Electron and Energy transfer in Polypeptides".
- Prof. C. Ferradini of the Laboratoire de Chimie Physique, School of Medicine Univ. of Paris (Rene Descartes), France, in the field of "Pulse Radiolysis Studies in Biochemical Systems". In 1982 the two laboratories submitted a research proposal on the subject. This proposal was granted (by the French counterpart). In 1984 Prof. Ferradini and Dr. M. Gardes did some studies with me in Columbus, Ohio (NATO grant to Dr. Gardes). This collaboration resulted with four publications. Later and with Prof. J.-P. Jay-Gerin we have studied the "Properties of the Solvated Electrons" which resulted in one publication. We have also collaborated on studies on "Radiation Induced Single Strand and Double Strand Breaks". This collaboration resulted in two publications.
- Prof. C. Houee-Levin of the Curie Institute and the Univ. of Paris-Sud (Orsay Campus) and Prof. P. Joles of the Univ. of Paris (Renee Descartes campus) on "Pulse radiolysis of Peptides and Proteins". This collaboration resulted in two publications.
- Prof L. Lindqvist of the Laboratory of Molecular Photophysics, Univ of Paris (Sud), Orsay, France, on "Photoionization of Tryptophyl Containing Polypeptides".
- Prof. B. Alpert of the Univ. of Paris (P. and M. Curie), Paris, France, on "Ligands and Radical Reactions with Alpha Hemoglobin.
- Dr. B. Hickel of the Pulse Radiolysis Laboratory, Saclay Nuclear Research Center, France on "Pulse Radiolysis Studies.

- Dr. P. Riesz of the Radiation Oncology Branch, National Cancer Institute, Bethesda, Md, U.S.A. on "The Mechanism of Action of Porphyrins, an Anticancer Drugs". This collaboration resulted with one publication.
- Dr. L. Gilles of the Department de Physico-Chemie, C.E.N-Saclay, France on "Physical and Chemical Properties of Dye Lasers".

6. PUBLICATIONS, COMMUNICATIONS AND LIBRARY TEXTS

6.1 Thesis:

1. Chemical Properties of Alginic and Pectic Acids in Aqueous Solutions (M.Sc. Thesis with Prof. G. Stein).
2. Effet de la Temperature et des Electrons sur la Reaction Graphite-Gas Carbonique, in partial fulfillment of the requirements for the degree of Nuclear Engineer, July 1958, C.E.A. Saclay, France.
3. Etude de la Decomposition du CO₂ sous l'Effect de la Decharge Electrique Silencieuse (These de Doctorat de l'Universite de Paris (Ph.D.) sous la Direction du Prof. Magat, 25 Octobre 1960, (T.H.) Jury: Prof. E. Bauer, Prof. M. Magat, Prof. M. Haissinsky).
4. Decomposition Radiolytique du Gas Carbonique (CO₂), These de Doctorat d'Etat, D. es. Sc., sous la Direction du Prof. M. Magat, Universite de Paris, 15 Novembre 1962.

6.2 Publications and communications.

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16. A. Apelblat and M. Faraggi, Dynamic Behaviour of a Stagewise Liquid-liquid Extraction process, J. Nucl. Energy, 20, 953 (1966).
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7. RESEARCH PROJECTS

7.1 Introduction

Two approaches to pulse radiolysis studies are possible when using this technique in areas like protein chemistry. The first, is connected with the damage created by radiation and in principal it deals with direct and indirect effects of radiation on substances of biological importance. This approach which may be called "classical", is the one used by radiation biologists. The second, is to use this technique as another physical method, producing reactive species (radicals, metal ions in unusual oxidation state(s)) permitting to follow their reaction with the biomolecule and those following the primary reaction in a long time span (from one microsecond to at least one minute). Moreover, using different detection methods (see later in section 7.2), the characterization of the transient species formed along the overall period of the reaction(s) is facilitate. In analyzing these results we can keep in mind problems that are of interest to biochemists or biophysicists (e.g. pK determination of histidyl residues, internal electron transfer reactions, stabilization of unstable radicals in proteins, the nature of the active site in proteins). We, therefore, build the systems to study accordingly. We can and should use data obtained by biochemists and biophysicists in areas related to our study (e.g. structure analysis, properties of biomolecules in solutions, other fast kinetics methods) and compare our results and those obtained by others. This approach envelop in essence the first approach. I have adopted the second approach which seemed to me wider and therefore more interesting. This is illustrated in many of my previous studies. The research program described in the following pages is a continuation of our earlier studies and tries to

further develop the ideas expressed in them. These are:

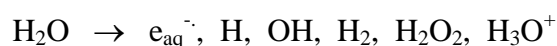
- (a) Internal electron transfer reactions in modified proteins, oligo and polypeptides, structural units of DNA and RNA. We are mainly interested in understanding four phenomena associated with these reactions; i) the nature of these reactions (can we invoke tunneling, can we describe these biomolecules as semiconductors), ii) how far can the electron travel in a biomolecule; iii) what are the factors that affect these reactions (redox potentials, charge effects, ligand effects and inductive effects); iv) do hydrogen bonds and/or side-chains affect these internal transfers?
- (b) Internal electron, and proton transfer reactions and conformational changes associated with these reactions in quinone containing proteins.
- (c) Reinvestigate (as we have done with peptides) radical reactions with structural units of DNA and the nucleic acid itself.
- (d) Previous studies on SOD led us to speculate that this protein is a general catalyst for the dismutation of free radicals. We would like to follow this idea.
- (e) The high potency of OH radicals and the presence of relatively high concentration of bicarbonate in cells lead us to think that the radical produced from this anion might have radiobiological implications.
- (f) The newly developed water soluble phthalocyanine, and their possible use in photo-irradiation therapy attracted our attention and we would like to follow their solution properties, radical reactions and photochemistry similar to the studies we have done with porphyrins.

In the following pages short descriptions of the pulse radiolysis technique and the above research projects will be given.

7.2 The pulse radiolysis method.

The pulse radiolysis method is the electron impact analogue of flash photolysis, with excitation and ionization by a short pulse of high energy electrons replacing the photo-excitation process. Since its introduction in 1960, the pulse time resolution has been progressively improved until it is now in the picosecond time range. The detection methods successfully used include spectrophotometry from the vacuum ultraviolet into the infrared, conductometry, E.S.R. absorption, polarography and resonance Raman. In our studies, 0.1 to 1.5 microsecond pulses are used routinely, and an ultraviolet-visible spectrophotometer is our detection device.

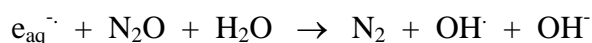
The high energy electrons (ca. 4 MeV from a linear accelerator or a Van der Graff machine or 1.8 MeV from a Febetron) introduced into dilute aqueous solution of a given biomolecule lose their energy predominantly by coulombic interactions with water. The products so formed are:



with yields (G values) of: hydrated electrons ($e_{\text{aq}}^{\cdot-}$), $G = 2.9$ radicals per 100 eV; hydrogen atoms (H), $G = 0.55$; hydroxyl radicals (OH), $G = 2.8$; H_2O_2 , $G = 0.75$; H_2 , $G = 0.45$; and Hydronium ions, $G = 2.9$.

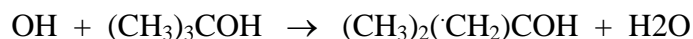
The primary radicals produced may be inter-converted, or removed by scavengers, permitting some choice of radical reactant for subsequent studies. Thus,

the hydrated electron can be converted into hydroxyl radical via:



therefore, in N_2O saturated solutions, OH radicals are the predominant species.

Hydrated electrons are obtained as a predominant reactive radicals by removing OH in the hydrogen abstracting reaction:



The t-butanol radical is neither a good hydrogen abstracting nor a good reducing agent and follow a fast radical-radical recombination reaction making it effectively, in most cases, invisible over the time range of the pulse radiolysis experiment. The aqueous radiolysis product, hydrogen peroxide is unreactive in most cases in the experimental time scale, and the hydronium ion is controlled with buffering.

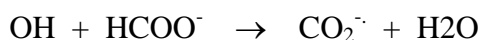
The hydrated electrons with a redox potential of approximately -2.8 V is a potent reducing agent with a broad absorption in the visible and the near infrared. In the absence of additional solutes, hydrated electrons decay by more than one path:

- i) combination with the proton to form an hydrogen atom;
- ii) combination with itself or with an hydrogen atom to form H_2 ;
- iii) in a slow reaction with water to form hydrogen atoms and hydroxyl ions and
- iv) by reaction with impurities in the solution.

The relative yields along these different decay pathways depends on the relative concentrations of the reacting species. Under the conditions of experiments normally performed in our laboratory, e_{aq}^- had a half life of approximately 5 to 10 microseconds. Because of its negative redox potential, the hydrated electron reduces a variety of organic and inorganic compounds; the rate constants of many of these reactions are known and compiled by the Radiation Chemistry Data Center, University of Notre Dame, Notre Dame, Indiana., U.S.A.

The hydroxyl radical, a potent and powerful oxidant with a redox potential of approximately + 2.3 V (at pH 7) reacts fastly with any electron donor and abstracts rapidly hydrogen from practically any CH_2 group in organic compounds. The radical absorbs only in the ultraviolet and therefore difficult to monitor directly, non the less, the reaction of OH with large number of compounds have been studied by competition techniques. A list of its rate constants have been compiled by the Radiation Chemistry Data Center.

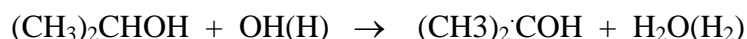
The OH radical is an intermediate in the formation of secondary reactants (both oxidizing and reducing agents). The formate radical, reductant with a potential of -1.9 V is produced in a formate containing solution via:



Since e_{aq}^- can be converted to OH radicals, and hydrogen atoms are converted to $CO_2^{\cdot -}$ by their reaction with formate, $CO_2^{\cdot -}$ is obtained as sole radical in N_2O saturated solutions. This radical has a strong absorption in the ultraviolet. Because of

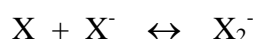
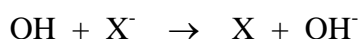
its lower redox potential, it is a poorer, thus more specific, reducing agent than e_{aq}^- .

The hydroxyl radical and the hydrogen atoms are also precursors of carbon centered alcohol radicals. Hydrogen abstraction reactions lead to the formation of the carbon centered radicals. For example the isopropanol radical is formed via:



The analogous ethanol and methanol radicals are produced in the same way. These three neutral radicals are reducing agents with potentials of -1.1 V, (isopropanol), -1.0 V (ethanol) and -0.8 V (methanol). They, therefore, constitute a series of neutral reducing species. We can obtain a wider emf spread in positively charged reductants. The hydrated electron reduces certain lanthanide(III) cations efficiently to produce: Tm(II), -1.9 V; Sm(II), -1.6 V; Yb(II), -1.2 V; and Eu(II), -0.4 V. Pulse radiolysis can also generate a variety of negatively charged species such as the superoxide radical or semiquinones with potentials varying from -0.6 to +0.1 V. Thus we can obtain rapidly a large number of reducing agent covering a wide range of redox potentials.

OH radicals can be converted to oxidizing species via their reaction with halides (X⁻):



With carbonate or bicarbonate to give $\text{CO}_3^{\cdot-}$, with azide ions to give N_3 radicals and with many others to yield different radical species. These radicals have lower oxidation potential, thus more specific oxidizing agents than the OH radical.

The concentration of radicals produced normally in our laboratory is in the range of 10^{-7} to 2×10^{-5} M that of the solute is normally one order of magnitude higher. This converts all primary second order reactions into a pseudo first order reactions which are easy to analyze kinetically. Because of the rapid initial reaction of the primary or secondary radicals with the solute, subsequent reactions (secondary, tertiary, etc.) are relatively easy to follow. Moreover, if solute concentration dependence is studied, distinction between first order (monomolecular, intramolecular) and second order (intermolecular) processes can be easily distinguished.

On the basis of studies with appropriate models of biomolecules constituents, one might predict that e_{aq}^- , OH, or any of the other radicals discussed earlier would react at a number of possible sites on the biomolecules; and, therefore, that the reactions of biomolecules with these radicals would be too complex for simple interpretation. Contrary to this expectation, experimental results have been generally opened to explanation. Let us consider the possible reasons for this unexpected simplicity.

It is a generality that the reaction of biomolecules with many radicals occur at or near the diffusion controlled limit. Consequently, when the protein to radical concentration ratio is sufficiently high (at least 10), the probability of more than one radical reacting with a single biomolecule is negligible because of its large size, and the resultant small diffusion coefficient, the biomolecule serves effectively (over the time span of most pulse radiolysis experiments) as an island, upon which one

reaction, or a sequence of reactions can proceed undisturbed by events on other biomolecules. Thus, cross reactions between secondary products are minimized, reducing possible complications. Nonetheless, a number of different reaction sequences may still be represented in the entire protein population, with one sequence on a set of molecules, a second on another set, and so on.

Differences in reactivities of the various constituents in biomolecules introduce an additional consideration. Reaction of a radical should be fast relative to the time required for that radical to diffuse into the biomolecule matrix, so that the initial reactions between pulse generated radicals and biomolecules should occur primarily at the protein surface, and not at internal loci which are shielded from the aqueous environment. Thus, partitioning between different possible reaction pathways should depend on the relative "concentrations" and intrinsic reactivities of constituents on the biomolecule surface. Constituents with low intrinsic reactivities would not compete effectively with those of high reactivity, unless the number of the former far exceeded that of the later. The tendency towards suppression of those reactions at less competitive constituents should, therefore, simplify the observed results.

A third factor to be considered is the selectivity engendered by the method of measurement employed. In most laboratories the appearance and disappearance of transients are monitored by optical absorbance changes. Thus, whether certain transients are detected depends upon the intensity of their absorbance, and the wavelength being monitored. Products of reactions without these properties will be difficult to detect. Thus, selectivity as introduced by the method of detection should also simplify the apparent results with, of course, a concomitant loss of information. This factor resulted in the fact that only a limited number of possible radical transients in biomolecules have been detected.

7.3. Internal electron migration in biomolecules.

The overall goal of this research is to determine the mechanism(s) of intramolecular long range electron transfer (LRET) reactions between two redox groups not in direct contact on the same protein molecule. To study the mechanism of LRET in proteins we need a protein with the following properties. There should be two unique redox participants to eliminate the potential problem of multiple processes. The two centers should be spectroscopically identifiable, and at a known, non-contact distance apart, deduced perhaps from X-ray crystal structure and other spectroscopic methods. We should be able to vary the distance between these two sites, to manipulate the difference between their redox potential (the driving force of the reaction), and finally, to alter the nature of the intervening polypeptide matrix. While a determination of the LRET rate dependence on the potential energy difference between the two redox centers is possible with a protein, the dependencies of LRET on the distance between the redox sites and the nature of the intervening polypeptide matrix cannot be investigated with a protein. This could be done however, with synthetic polypeptides.

This research program suggests to develop a theory for LRET assuming a semiconductor-like behavior of a polypeptide chain and to obtain relations between the rates of electron migration, and i) the distance between the redox centers, ii) the driving force of the LRET reaction, and iii) the effect of temperature. We also plan to

experimentally investigate: i) the effect of the driving force in modified (creation of a second redox center) proteins, ii) the effect of amino acids side chains in a polypeptide backbones (rigid rod-like structure constructed from proline) and iii) the effect of hydrogen bond network (alpha helix structure constructed from glutamic acid) on LRET. The last two proposals are connected with recent suggestions that electron migration may be facilitated by the presence of amino acid side chains and/or along the axis of the polypeptide alpha-helix. We suggest to examine these attractive proposals. We shall consider the effect of the amino acid side chain first. While there has been informal speculation and indirect findings (including one from my laboratory) that electron migration may be facilitated by the presence of these side chains or alpha helix structure, I know of no unambiguous evidence to support such attractive proposals.

The question of collisionless electron transfer over long distances has taken on new interest with the recent realization that such intramolecular migration may be quite common. LRET could be important in the mechanisms of protein mediated physiological redox reactions such as those that occur as part of respiration, and photosynthesis. An understanding of this transfer in macromolecules may have a bearing on the question of the mechanism of protein and DNA damage by ionizing radiation. Finally, an understanding of LRET may have a novel applied aspect in the field of molecular electronics commonly known as the biochip.

The biochip industry is considered to be a multibillion dollar enterprise. Computer and other high technology establishments and governmental agencies in the U.S, Japan and the U K. are investing in this research. The first (of four) biochip developmental stages are partly in production. For example, the Japanese have recently announced the fabrication of a biological PM junction. It is expected that the devices resulting from the first stage will be as much as 1000 times denser in information content than the most advanced silicon chip. The final goal of the biochip industry is to provide a computer based on a superconducting wave transmitted along a chain of atoms. One of the major problems in the development of such a device is to find biological or chemical system that will transport and store energy (information). The protein chain is considered to be one of the most promising systems. Some of the phenomena associated with the development of this system are: i) Proteins as semiconductors. ii) Long range energy transfer iii) Intramolecular energy transfer iv) Intermolecular energy transfer.

7.3.1. Biopolymers as semiconductors. A theoretical study

The oft speculated occurrence of intramolecular Long Range Electron Transfer (LRET) has now been observed in a number proteins and small molecular systems. Different approaches have been undertaken to provide evidence for intramolecular LRET in proteins. These were coordinative chemical modification to bind redox active center to specific peptide residue on the surface of the protein (Ru(III)(NH₃)₅ to histidyl imidazole). With these derivatives LRET between Ru(II) (obtained by either pulse radiolysis or flash photolysis) and the active site of the proteins were studied. In another approach hemoproteins reconstituted with photo-active heme group to investigate LRET in metal hybrid hemoglobins or stable complexes of cytochromes have been used. The third, involved analysis of intramolecular electron transfer in proteins containing two redox sites per protein

molecule. Intramolecular LRET across oligoglycines, (from $n=0$ to $n=3$) as spacers has been studied. The results showed an unexpected small decrease in the intramolecular rate constant for $n=0,1,2$ (from $7.3 \times 10^4 \text{ s}^{-1}$ to $2.4 \times 10^4 \text{ s}^{-1}$) and an increase for $n=3$ ($3.2 \times 10^4 \text{ s}^{-1}$). This behavior has been attributed to the flexibility of the oligoglycines in bringing the donor and acceptor to close proximity. In another study oligoprolines as spacers and Ru(II)/Co(III) and Os(II)/Co(III) as redox couples has been used. The rates observed with the first redox couple were very slow (rate constants of the order of 10^{-5} s^{-1}) and during this time period (hours) conformational changes (trans-cis isomerization) could bring the donor and acceptor to close proximity. These slow rate constants were attributed to the fact that the driving force of the reaction (the difference in the $E_{1/2}$ for the Ru(II)/(III) and the Co(III)/Co(II) couple) is +0.5 V (endothermic). In order to speed up LRET the driving force of the reaction was increased to -0.27 V by replacing the ruthenium ion by osmium ion. This replacement enhanced the reaction by a factor of 106. With the Os(II)/Co(III) system the exponential variation of the LRET first order rate constants as function of the number of prolines in the spacer was clearly observed.

Most of these studies and those using other small molecular systems and model compounds were interpreted by the Marcus theory as modified by Hopfield and Jortner for non-adiabatic (tunneling) electron transfer reactions. This model assumes that LRET reactions involve a weak coupling between the reactant and product surfaces. Therefore, the probability that any reaction that reaches the nuclear configuration at the classical turning point is less than one. This probability can be formulated as a problem of electron tunneling. The superexchange model offers a different approach. This model allows for ready charge propagation via either electron or hole tunneling between nearest neighbors. Both models predict an exponential dependence of the rate of LRET on the distance between the redox sites (R).

$$k \propto \exp(-\beta R)$$

The question now is to determine the value of b . According to the modified Marcus theory b is proportional to the square root of the barrier height (electron binding energy). However, its exact value is not known. Jortner sets $b = 2$ while Hopfield suggests $b = 1.4$. In the superexchange model X is proportional to the natural logarithm of the barrier height (Miller suggested $b = 1.1$). Therefore, the difference between these models resides in the importance of the exponential dependence. The modified Marcus theory predicts a more pronounced effect. The Marcus model predicts also an inversion in the dependence of the redox potential difference (driving force) between the two redox centers.

An alternative mechanism for intramolecular ET in biopolymers (proteins and polypeptides) is to assume that just as in polymers the interaction of a polymer unit cell with its all neighbors leads to the formation of electronic bands, valence (VB), conduction (CB), and an energy gap (E_g) between them. Therefore, electron transfer across a biomolecule chain is controlled by the chemical nature of the chain. The property that is important to its electron transfer is the electronic structure of the polypeptide backbone. This can be viewed chemically because of the partial double bond nature of the C=N groups and the dipoles on the C-O (negative) and N-H (positive) groups. If electron transfer is to occur along a polypeptide chain, electrons from the C=N groups must interact and cross a gap consisting of a saturated (sp^3)

carbon atom. The possibility of internal ET (electron transfer) pathways involving peptide backbone and hydrogen bonding in proteins was discussed in the past. The conclusions were that the large energy gap (-3 eV in polyglycine) between HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) prevent electron mobility through the polypeptide backbone. This gap could be lowered if we think of the amino-acids side chains as impurities (dopants) in the main peptide chain. Thus, this model assumes that the polypeptide backbone has a semiconductor-like properties. Another pathway for LRET that has been suggested involves the hydrogen bonding network, found for example in alpha helices or beta plated sheets. This network can form a conduction band with a small energy gap between the HOMO and LUMO (-0.3 eV). Recently, we have started a theoretical study assuming a on the semiconductor-like properties of polypeptides. Using recent development in the theory of semiconductors we wish to establish the distance dependence, (we expect that with this model the rate will depend only weakly on distance rather than exponentially) driving force effect, and temperature effect on LRET. This study is performed in collaboration with the solid state group (Prof. J. P. Jay-Gerin) of the university of Sherbrooke.

7.3.2 Protein long range intramolecular electron transfer reactions (1985 BSF & NIH projects).

The question of collisionless electron transfer over long distances has taken on new interest with the realization that such intramolecular migration may be more common than previously envisioned and claimed by studies using pulse radiolysis. Collisionless transfer has been studied in various model systems, and there are recent reports, including some from our laboratory, of PIET in a number of different proteins. Clearly PIET might be important in protein mediated physiological redox reactions that occur, for example, in respiration and photosynthesis. It has been generally assumed that donor and acceptor groups must be in close proximity during an electron transfer between two proteins. In those cases where a reasonable argument could be made that at least one of the redox centers is buried within the protein matrix, formation of a protein-protein complex with an induced conformational change to permit redox center contact has been generally accepted. When the redox center is located even partially on the protein surface, and so, is possibly accessible to another electron donating protein, a problem still arises if the newly reduced site acts in turn as an electron donor to a protein further along an electron chain. Then, one must postulate either a flip-flop motion of the acceptor-donor protein between its fixed partners, or two association-dissociation processes. These theoretical difficulties vanish if we can invoke LRET.

Our pulse radiolysis studies on the reduction of imidazole groups attached to proteins (106,107,110,136), as previously outlined (see chapter 4), suggest an additional novel aspect of LRET. It is generally accepted that the distribution of charged groups on a protein s surface affects the interactions of that protein with other molecules. By extension, the non-uniform distribution of charged and dipolar groups both on and within a low dielectric protein interior must surely generate a potential energy "surface" within the macromolecule. This potential surface would be expected to affect not only intramolecular electron movement, but other biological functions of a macromolecule such as the catalytic mechanism of an enzyme. In so far as that

surface may be reflected in the PIET, the study of these long range transfer processes will yield information concerning a property of proteins that has not been subjected to experimental probing to date. Before we can fruitfully invoke PIET as either a theoretical concept or a practical tool, we must have some understanding of the phenomenon's mechanism. The aim of our research is to gather data to be used in a discussion of that mechanism.

Our proposed approach to the study of PIET is to choose proteins containing one SH group exposed to the solvent (water) and a metal ion (Fe(III)) associated with the enzymatic activity of the protein. Proteins fulfilling these requirements are: α -Methemoglobin (human), a mutant Metmyoglobin and Hemerythrin.

Our first task is to introduce a second distantly located site, at the lone cysteine (-SH) of each protein. The cysteine will be either alkylated with an EDTA analogue with which lanthanide ions will be chelated, or blocked as a mixed aromatic disulfide. We shall initiate the experiment by pulse radiolytic introduction of either the hydrated electron (e_{aq}^-), the formate radical (CO_2^-), or other carbon-centered radicals for rapid reduction of one or both redox sites on the modified protein, and shall look for the LRET during the subsequent redox equilibration between the two centers. Intramolecular electron transfer reactions should be identifiable either indirectly as a protein concentration independent kinetic redox reaction at one of the two sites, or from the direct observation of a first order precursor-product reaction between the two sites. We shall then look for rate changes in the LRET that are dependent upon the following protein modifications:

- i) different chelated lanthanide ions;
- ii) different aromatic substituents in the mixed disulfide;
- iii) changes in the amino acid residues that are physically located between the two redox centers.

With these modifications, we hope to determine the dependence of the LRET rate on the difference in redox potential between the two centers, on their distance apart, and on the nature of the intervening protein matrix.

As background for this project, we shall also study the one electron redox reactions between the disulfides and lanthanides, the redox potentials of chelated lanthanides and aromatic disulfide radical anions, and the reactions of each of the unmodified proteins with e_{aq}^- , CO_2^- , and carbon-centered radicals.

7.3.3. Long range electron transfer reactions in polypeptides. (BSF & NIH projects 1985/86).

Recently, evidences have been accumulated showing that long range electron transfer processes occur in peptides and proteins. We now need to learn more about this process with its important implication for the function of biologically significant protein redox reactions, the properties of macromolecules and the migration of electrons in non-conductors in general.

We suggest to extend our studies on electron migration across biopolymers and investigate the assumptions:

- i) amino acids side chains may act as impurities ("dopants") in a main polypeptide chain and facilitate LRET, and

iii) hydrogen bonding network facilitates LRET.

To search for long range electron transfer along a polypeptide chain we shall attach an electron donor onto one end and a proto-electron acceptor onto the other end of the polypeptide chain. We shall oxidize the proto-acceptor with a pulse radiolytically generated radical, and then look for subsequent electron transfer from donor to acceptor. The long range electron transfer will be identified in terms of the kinetic order associated with this transfer, and the dependence of the intramolecular rate constant on the length and secondary structure of the intervening polypeptide chain. Our choice of the experimental system is based on previous results by Prutz et al., who oxidized a tryptophan on one end of an oligoglycine chain with the azide radical (N_3), and then, observed subsequent reduction of the indolyl radical with a concomitant one electron oxidation of a tyrosine attached to the other end of the chain. Since both indolyl and phenoxy radicals absorb strongly in the visible region, this electron transfer is easily monitored.

There has been suggestions that electron migration may be facilitate by the presence of amino acid side chains and/or along the axis of the polypeptide alpha-helix. We suggest to examine these attractive proposals. We shall consider the effect of the amino acid side chain first.

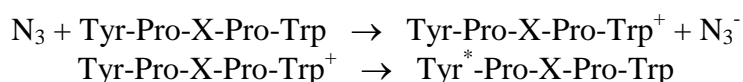
7.3.3.1. The effect of amino acid side chains on LRET

We propose to study LRET across a rigid (rod-like structure) in a series of peptides, tyrosinyl-(prolyl)_n-tryptophan (Tyr-(Pro)_n-Trp), (Trp-(Pro)_n-Tyr) of different length (from n=1 to n=5), and the effect of side chain amino acids "doped" at various locations along the polyproline chain. We suggest to study both types of peptides (e.g. (Tyr-(Pro)_n-Trp), and (Trp-(Pro)_n-Tyr)) since the position of the terminal carboxyl (or amino) group may affect (by the location of its charge) the rate of the electron migration. For comparison, some experiments will be carried with "doped" polyglycines which have a random coil structure. The side chain amino acids will include:

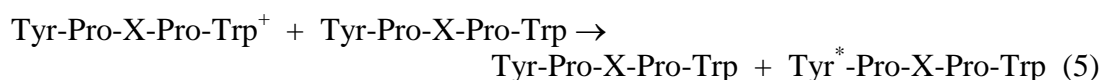
- i) an aromatic side chain (phenylalanine) with its low lying, empty, π orbitals;
- ii) a sulfur containing side chain (methionine) with its low lying, empty 3d orbitals;
- iii) charged side chains (aspartic acid, lysine, arginine);
- iv) serine, and;
- v) threonine.

All these "dopants" have low lying, nonbonding orbitals that can assist in closing the energy gap between the valence and conductivity bands in the polypeptide backbone. For example, if LRET will be followed with the starting peptide Tyr-(Pro)₂-Trp, each of the "dopants" (X) could be placed between the two prolines (Tyr-Pro-X-Pro-Trp).

The electron migration will be initiated by oxidizing tryptophan with an azide radical, generated by pulse radiolysis, and we will follow the reduction of the indolyl radical with the concomitant oxidation of the phenol ring.



This first order electron transfer is easily monitored, since both indolyl and phenoxy radicals absorb strongly in the visible region. The second order component of this electron transfer could be measured as a concentration effect:



Previous studies have shown that the second order rate constant of the above reaction (5) is of the order of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction TyrH and Trp⁺. This value decreases with the peptide length, probably due to a diffusion effect (Klapper and Faraggi, to be published). Since the radical concentration in our experiments is of the order of $5 \times 10^{-6} \text{ M}$ and that of the peptide of the order of 10^{-4} M , corrections for the contribution of intermolecular reaction should be made when the overall rate of electron transfer will be 7 s^{-1} or less.

The first order rate constant of the LRET reaction obtained will be compared with that for Tyr-(Pro)₃-Trp. The comparison between these tripeptide spacers should tell us if the presence of the side chain amino acid (X) has an effect on LRET. However, since the "dopant" amino acid may introduce flexibility to the rigid spacer experiments with glycine replacing the "dopant" will be also performed. Certainly we do not propose to study all the possible compounds (we have counted 920 different of them). We shall perform however, a systematic study with a short spacer (the one given above) Which are less expensive and easier to synthesize. Once we shall find (we hope) the amino acid side chains that enhance LRET mostly, the other spacers will be studied with those only.

7.3.3.2 The influence of the alpha-helix structure

To test that electron migration may be facilitate along the axis of an alpha-helix we suggest to use poly-L-glutamate (PGA) as spacer. PGA is known to be in the alpha helical structure at Ph below the carboxyl pK_a, and in a random coil structure at a pH above this pK_a. Here again tyrosine and tryptophan will be attached to each end of the spacer (Tyr-(Glu)_n-Trp). The electron migration will be initiated by oxidizing tryptophan with the azide radical, after which the reduction of the indolyl radical with the concomitant oxidation of the phenol ring will be followed.

In these experiments using PGA the progression distance in the alpha-helix is 0.15 nm per residue with one complete turn of 0.54 nm length every 3.6 residues. Since a stable helix most probably requires a 4-5 residue stretch, we can vary the distance between the redox groups at the two ends of the structurally rigid helix from ca. 0.7 to 1.5 nm with a PGA spacer of 4 to 9 residues. The PGA helix thus becomes a "ruler" permitting us to determine how the LRET decreases as the distance between redox centers increases. Additionally, a sharp change in the LRET, were it to occur somewhere between the introduction of 2 to 5 intervening glutamates, would indicate possible alpha helix facilitation of the electron transfer process. WE can also test for facilitation with the control polypeptide insert, poly(hydroxyethyl)-L-glutamine (PHEG), PHEG is a water soluble randomly coiled neutral polymer that would be a

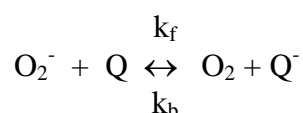
better control than the charged poly-L-glutamate random coil. Since the ORD of the PGA alpha-helix is well known, it should be possible to verify the secondary structure of the polymer under various experimental conditions. Analysis of conformation and flexibility of the polypeptides presents a challenging problem. We think that X-ray analysis (definition of the conformation in the crystalline state), high resolution NMR (conformation and dynamics in dilute solutions), and other spectroscopic probes such as CD, ORD and tryptophan fluorescence will give us enough information on the conformation of the polypeptides in aqueous solutions.

Finally, I propose to alter the redox potential of the phenoxy/phenol couple by substitution into the tyrosine side chain. At fixed distance between the redox centers an increase in the redox potential difference should result in an increase in the LRET rate constant. However, I would not expect in this system to generate the potential differences needed to reach the inversion point in the rate versus driving force curve predicted by the modified Marcus theory for non-adiabatic reactions.

7.3.3.3. Additional Experiments

The experiments described above are based on standard chemistry and technology, but successful interpretation of the results will require additional information. I have proposed to determine the dependence of the LRET on redox potential difference between donor and acceptor sites (driving force of the reaction). This presumes we know or can determine the redox potential of the proposed acceptor and donor pairs. Other than estimates of the tyrosine phenoxy radical, +0.8 V, and the tryptophan indolyl radical, +0.6 V, there is no redox information available peptides containing these amino acids and on modified tyrosines. An important task is, thus, the determination of redox potential in small model compounds. Potentials for organic radicals are difficult to obtain, since these are so unstable, with rapid decay by radical-radical recombination or cleavage. To determine the redox potential in such cases we can use either fast electrochemical techniques or pulse radiolysis. The first, is limited to relatively stable radicals (with half lives of the order of a millisecond) and needs a fast computer controlled data acquisition system.

The second method, pulse radiolysis, generates the radical of interest in the presence of a second one-electron redox couple for an equilibration that is faster than the radical loss. For example, we have recently determined the quinone/semiquinone EMF of two phenantroline diones and Methoxatine. The superoxide ion, generated by pulse radiolysis, initiate the equilibrium reaction:



The decay to the final equilibrium was monitored from the absorbance of the semiquinone in the visible. The constants k_f and k_b were obtained from the pseudo-first order rate constant dependence on oxygen and quinone concentrations, and the equilibrium constant from the concentration dependence of the equilibrium absorbance. The one step mechanism was verified by the close similarity of K_{eq} and k_f/k_b . Given the known potential of the O_2/O_2^- couple (-0.155 V vs. NHE, pH 7, 1 M

O₂ standard state) we obtained the potential of the Q/Q⁻ couple in these systems (0.110 to 0.030 V depending on the orthoquinone). The three requirements for the success of this approach are:

- i) sufficient stability of the radical over the microsecond time range in which radical decay is minimum;
- ii) a reference redox pair that reacts rapidly with the radical and has
- iii) a midpoint potential near that of the radical under study.

The radicals in this proposal are sufficiently stable in our hands. Reasonable reference pairs with reversible or near to reversible behavior at electrode surfaces should be fast enough reactants. Finding a reference pair with the correct EMF will be hunt-and-peck. For example, for our radicals it seems that IrCl₆^{2-/3-} (E_{1/2} = +0.89 V), Mo(CN)₈^{3-/4-} (E_{1/2} = +0.76 V), and the water soluble ferrocenes seem most suitable. A list of radicals with potentials ranging from +0.99 V (benzoquinone) to -0.93 V (NAD⁺) is given by Swallow.

7.4. Long Range Electron And Energy Transfer In Peptides. (BSF, MRC, NIH & DOE 1991 PROJECTS).

7.4.1. Introduction

7.4.1.1. Current Status of Long Range Electron Transfer Theory.

The discovery of Long Range Electron Transfer (LRET) in proteins has led to a flurry of activity, reflected in the many reviews of the past decade. The LRET process has been explained in terms of the semi-classical Marcus theory. Briefly, there is a through-space electron tunneling between two redox centers (donor, D, and acceptor, A,) held together as harmonic oscillators. An important result of the theory is an analytic expression for the electron transfer rate constant, k_{ET}:

$$k_{ET} \propto \exp[-\beta(r_0 - r_{DA})] \cdot \exp[-(1 + \Delta G^0)^2/4RT]$$

Three important variables in this equation are:

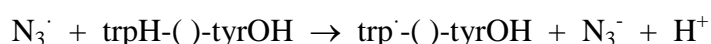
- a) The nuclear reorganization energy or work required to excite the electron to a product with the nuclear (including solvent) geometry of the reactant;
- b) ΔG^0 , the overall equilibrium free energy; and,
- c) α , and β distance damping factor characteristic of the wave function overlap required for tunneling.

Three important predictions follow from this equation. First, k_{ET} should increase to a maximum and then decrease as ΔG^0 becomes increasingly negative and all else remains constant. This inversion is due to the arithmetically even exponential term $(1 + \Delta G^0)^2$. Second, k_{ET} should depend on the nature of the solvent since it must also account for solvation changes around the reacting center. Third, beyond a minimum distance r₀, the electron transfer rate constant should decline exponentially

with increasing distance, r_{DA} , between donor and acceptor. Each of these predictions has been verified in one or another system. Thus, the LRET k_{ET} declines exponentially with increasing distance over the oligoproline peptide, and Axup et al. have data consistent with an exponential decline in a single protein system. The inverted region has been reported by McClendon in a photoinduced electron transfer between mixed metal heme centers in the Cytochrome *c/b* complex, by Miller et al. between organic redox centers separated by a rigid saturated tetracyclic hydrocarbon spacer, and by Gray and coworkers. Miller and coworkers have also reported the predicted solvent effect on the LRET rates. But even with the qualitative success of Marcus theory, there is another mechanism under active discussion (e.g., 4). A system of nonconjugated bonds connecting donor and acceptor need not be only a scaffold for through-space tunneling via the overlap of the redox center orbitals. Electron transfer could occur by a through-bond process in which the orbitals of the communicating molecular framework mix with those of the redox couple. There is both theoretical justification, developed in terms of superexchange coupling, and experimental support for this suggestion. In order to deal logically with the question of whether and/or how a protein's structure affects its physiological LRET process we must establish whether electron transfer is through-bond, through-space, or some combination of both.

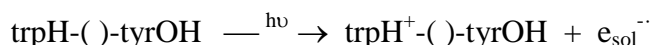
7.4.1.2. The Flash Photolysis and the Pulse Radiolysis Techniques.

Laser flash photolysis and pulse radiolysis have been the primary kinetic techniques for LRET experiments. In flash photolysis a light pulse with known energy (~6 eV) and duration ($t \geq 100$ fs) is delivered to the solution and excites directly one or more *solute* species. The resultant photoexcitation and/or photoionization are monitored most often by spectral changes in the UV-visible region. In pulse radiolysis an electron pulse with known energy (≥ 2 MeV) and duration (100 ps to 1500 ns) is delivered to the solution and excites the *solvent*. The primary radicals produced because of this solvent excitation are then used to establish the LRET chemistry on the solutes. In this case also, the UV-visible spectrophotometer remains the most popular detection device. In the past our preferred technique was pulse radiolysis, which, though not familiar to many, has been the tool in many LRET studies. Because of space limitations, we shall not describe the basis of this technique. Suffice to say that with accelerator generated high energy electrons we can obtain in less than 1 ms any one of a number of free radical reactants. For the purposes of this proposal, only the azide radical (N_3^\cdot) will be under discussion. Hydroxyl radical (OH^\cdot) produced in the pulse radiolysis of N_2O saturated aqueous solutions containing the azide anion reacts with the N_3^- to generate the azide radical, a 1-electron oxidant. The N_3^\cdot experiment starts with the preferential oxidation of one site on a molecule. Intramolecular electron transfer then follows. The system we have been using was developed by Prutz et al. with peptides that contain one tyrosine (tyrOH) and one tryptophan (trpH). N_3^\cdot oxidizes the trpH side chain with a bimolecular rate constant of $\sim 3 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. Since tyrOH reacts roughly 30 times less rapidly, the trpH oxidation reaction is preferred. The trpH radical (trp $^\cdot$) in turn oxidizes the tyrOH side chain to the phenoxy radical (tyrO $^\cdot$).





In flash, now usually laser, photolysis the photon is absorbed specifically by those solute molecules with the appropriate absorption band and with an efficiency dependent on the extinction coefficient of the solute at the irradiation wavelength. In our trpH/tyrOH system the electron transfer process is established if the light absorption results in a ground state electron acceptor due to photoejection.



The amount of oxidant/reductant obtained from a particular solute depends on the characteristic quantum yield, ϕ , defined as the number of oxidant/reductant molecules produced per photon absorbed into the system. For this reaction, with 266 nm light, $\phi < 1$ since tryptophan triplets are formed in competition with the radical cation oxidant. The elimination of the $e_{\text{sol}}^{\cdot-}$ is done with oxygen. After a very fast release of a proton the photoionization reaction will be followed by the electron transfer reaction (see above). Since both $\text{trp}^{\cdot-}$ and $\text{tyrO}^{\cdot-}$ absorb strongly in the visible, it is easy to show that:

- i) tyrOH oxidation to $\text{tyrO}^{\cdot-}$ and $\text{trp}^{\cdot-}$ reduction to trpH occur simultaneously;
- ii) electron transfer is quantitative; and
- iii) there are two isosbestic points in the time dependent absorption spectra.

Hence, the electron transfer reaction has no kinetically significant reaction intermediate, and the identities of electron donor and acceptor are unambiguous. A further major advantage to this system lies in its biological significance. $\text{tyrO}^{\cdot-}$ may be a reaction intermediate in the photosystem II light reaction, and in at least two important enzymes ribonucleotide reductase and prostaglandin synthase. Most recently a neutral tryptophan indolyl radical has been observed in the H_2O_2 /Cytochrome-c Peroxidase complex, and it has been proposed that the electron transfer reaction may be important in this enzyme's catalytic mechanism.

7.4.1.3. Long Range Electron Transfer in Proteins.

It is believed that Efficient LRET between a donor and its distant acceptor in proteins are found in:

- i) has biological significance in photosynthesis, respiration and enzyme catalyzed redox reactions;
- ii) may be important in mediating free radical damage initiated by ionizing radiation and oxygen assault; and
- iii) may occur in the radical processes associated with chemical carcinogenesis and cellular aging.

Although presumably important in other biological molecules, with few exceptions, reports on biochemically based LRET have dealt with peptides and proteins. (There have also been numerous important studies involving spacers that are not peptides, nucleotides, etc.; e.g., the results obtained with polycyclic spacers.

As mentioned earlier, the available protein LRET data are qualitatively consistent with the Marcus theory when comparing results obtained with a limited number of proteins. However, the expected correlation breakdown when considering the data collected from many protein systems. Thus, there must be additional important determinants of the LRET process in proteins; a conclusion that is supported by the few observations of LRET pathway and redox site specificity, by the observation of Sakata et al. that electron donor and acceptor orientation affects the LRET rate, and by our observation that net electron transfer along a peptide chain can affect the LRET rate. Moreover, there have been theoretical considerations of the possible role played by the intervening protein matrix in the LRET mechanism. This then leads to further speculations; e.g., whether LRET is promoted by hydrogen bonds, whether a secondary structural feature such as the α -helix or β -sheet influences LRET, etc. To establish whether and how the intervening protein matrix alters the LRET process is important with respect to another question. Could evolutionary pressures have led to enhanced or diminished electron transfer along specific paths and between specific groups in proteins, thereby directing a protein into one or another specific biological electron transfer function? The experiments of this proposal are designed to look for possible LRET controlling factors in the various structural elements of proteins and peptides.

7.4.1.4. Why Study Long Range Electron Transfer in Peptides?

While developing our work in protein LRET, we turned to LRET studies in peptides. There is a major advantage to peptides because of the ease with which they are manipulated synthetically. It is thus easier to isolate the various structural factors that might influence the electron transfer process. Our experiments to date serve as examples of what should be possible.

- i) Because of the proline ring rigidity, the oligoproline chain ends are held scrupulously apart, and each inserted proline residue increases linearly the distance between the two terminal amino acids tyrosine and tryptophan. Thus, the oligoproline spacer is well suited for studying the dependence of electron transfer rates on distance.
- ii) Currently, there is no experimental data in the literature concerning the effects of protein secondary structure on LRET rates. Based on the reported syntheses of small α -helical peptides, we have started exploring the effect of the α -helix on electron transfer.
- iii) The introduction of unnatural amino acids into a test system allows for finer structural modulations.

Although inserting such residues into proteins is possible, the production of "unnatural" protein mutants in sufficient quantity for extensive experimentation is not yet feasible. On the other hand, we have already started using unnatural amino acids in our peptide LRET studies.

7.4.2. Objectives And Expected Significance Of The Research

LRET occurs in both peptides and proteins, and it is an obligatory event in

photosynthesis and other protein mediated processes. Thus, there is an interesting question of whether and/or how protein structure controls LRET. To answer this question one can both appeal to theory - LRET is generally discussed in terms of Marcus theory and seek out new experimental results - our recent peptide data are not entirely consistent with that theory. It is this interplay between experiment and theory that will lead to the answer for this question, and our goals are:

- i) to collect additional data that either support Marcus theory or contribute to the experimental foundation for its modification; and
- ii) by investigating those factors that affect LRET in peptides, to determine how protein structure may impose pathway and redox site specificity on the LRET process.

The peptides proposed for these studies have two obligatory features: electron donor and acceptor sites that are not in contact with one another, and an intramolecular LRET process that we can initiate by the fast kinetic techniques of flash photolysis and pulse radiolysis. The objectives of this proposal are enumerated next.

7.4.2.1. Is LRET in Peptides Through-Bond or Through-Space?

LRET is generally considered a tunneling process, and there are at least two possible tunneling mechanisms: the first is electron transfer through direct redox center orbital overlap (through-space); the second involves a mixing of the redox center orbitals with those of the communicating molecular framework (through-bond).

We propose to test for one or the other (or a mixture of both) with a rigid hairpin peptide. The hairpin turn will be three residues of the amino acid 1-amino,1-carboxycyclopropane (acc) and the tines two stretches of diethylglycines (deg). Because of the bulky deg side chains there can be no H-bonding between the two tines. The two redox centers, tyrosine (tyrOH) and tryptophan (trpH), will be at the two ends of the peptide in order to determine whether electron transfer occurs along the polypeptide chain or across the hairpin gap.

7.4.2.2. Does the α -Helix Affect LRET?

We shall investigate the effects of the α -helical structure on LRET by placing the tyrOH and trpH redox centers within a leucine zipper peptide fragment modeled from the yeast transcriptional activator GCN4. This peptide forms parallel dimers with > 90% helicity at 0°C. As a second system we shall investigate LRET across peptides that form monomeric helices. Large differences in the LRET rate before and after chain melting will signal a structural effect.

7.4.2.3. How Does a Fully Extended Peptide Chain Affect LRET?

We shall investigate LRET across oligo-peptides made of deg or hydroxymethylserine (hms), both of which should assume fully extended conformations.

7.4.2.4. How Do Inter- and Intra-chain Electron Transfer Rates Compare?

LRET occurs between protein subunit chains. We shall compare intra- to inter-chain LRET with synthetic analogs of the leucine zipper Cfos-Cjun heterodimer.

7.4.2.5. What is the Effect of the Equilibrium Driving Force (ΔG^0) on LRET Rates?

Theory relates LRET rates with the ΔG^0 and distance between electron donor and acceptor. We also have preliminary evidence that the direction of the electron transfer along the peptide chain affects the LRET rate. We shall construct oligo-proline and oligo-hms peptides separating donor/acceptor amino acid centers with redox potential differences ranging from ca. 0 V (tyrOH and tryptoline-3-carboxylic acid) to ca. 0.4 V (trpH and ferrocene). With these peptides we shall investigate the effects of both redox potential differences and distances on LRET rates.

Gray and Malmstrom have recently suggested that protein structure can control electron transfer by affecting nuclear reorganization energies. The experiments we propose here explore a complementary premise -the structure of the intervening protein matrix controls electron transfer.

7.4.2.6. What is the Effect of the Solvent on LRET Rates?

Theory relates also LRET rates with the reorganization parameter, which is equal to the energy difference between the reactants' and products' free-energy surfaces at the reactants' equilibrium nuclear configuration when $\Delta G^0 = 0$. λ contains two major contributions: λ_{in} associated with the generally fast changes in the intramolecular bond distances and angles; λ_{out} is associated with the generally slow changes in the polarization of the surrounding medium (solvent polarity). Pulse radiolysis does not normally allow experiments with other solvents than water because our knowledge of the radiation chemistry of water and aqueous solutions is much better than that of organic solvents. However, in flash photolysis experiments only the solute interacts with the photons and no chemical changes is expected in the solvent.

7.4.2.7. Triplet-Triplet Energy Transfer and LRET.

The idea to compare triplet-triplet energy transfer rates to those of LRET follow the results and suggestion of Closs et al.. Dexter has shown that, although the energy transfer process is dipole forbidden it can occur by an exchange mechanism which is formally a simultaneous double electron transfer. Since no charge buildup occurs during the triplet-triplet transfer (electrons are simultaneously exchanged between donor and acceptor), λ_{out} and solvent dynamics which may dominate LRET, can be neglected in energy transfer. Therefore, a better information on λ (the distance damping factor, see Marcus equation, could be obtained. Closs et al. found that, for a common series of spacer molecules, and therefore the same electronic coupling terms, $\ln(k_{ET}) = 2\ln(k_{EX})$. Bashkin and McLendon applied this concept to the protein

complex Cytochrome *c*: Cytochrome *c* Peroxidase and found a β value of 0.85 much lower than that of 1.2 found from LRET experiments.

7.4.3. Methodology And Plan Of Operation

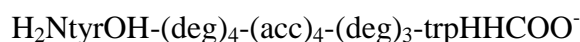
7.4.3.1. Previous Work - Peptides

In summary, the pulse radiolysis technique together with the tyrOH/trpH system has so far yielded a number of interesting results. Among these are the first experimental evidence for through-bond LRET, an LRET distance dependence that challenges current theory on the LRET mechanism, and an interesting start on the question of whether secondary protein structure affects LRET. Finally, tyrO \cdot and trp \cdot radicals have been implicated recently in biological reactions. The additional information we propose to gather may prove invaluable in understanding the mechanisms of these reactions and the many more that will be uncovered over the next decade.

7.4.3.2. Experimental Design and Methods

(a) ***OBJECTIVE 1: Is LRET Through-Bond or Through-Space?***

The results obtained with the oligoproline spacer suggest that LRET in peptides is a through-bond process. We propose a more rigorous test of this proposal with the synthesis of a hairpin structure in which tyrOH and trpH are far apart along the peptide chain but close to one another in space. The construction of this hairpin is based on two observations: the crystal structures of 1-amino, 1-carboxycyclopropane (acc) tripeptides have dihedral angles consistent with formation of distorted 3_{10} helices or type I β -bends, and oligo-peptides of diethylglycine (deg) are fully extended because of the ethyl side chain steric interactions. With the molecular modeling program Biograf, we have found that the peptide:



has an energy minimized hairpin structure with a β -turn stabilized by three hydrogen bonds in the $-(\text{acc})_3-$ loop; the fully extended deg fragments form the two tines of this hairpin. In this structure the aromatic residues at the two ends of the chain are close but not in Van der Waals contact; and because of the bulky deg side chains, there is no H-bonding between the two tines.

		TABLE I	
		peptide rate relative to peptide II	
(I)		through-bond	through-space
acc-(deg) ₂ -trpH	I	faster	--
acc-(deg) ₃ -tyrOH	II	--	--
(II)	III	--	slower
acc-(deg) ₂ -trpH			
acc-(deg) ₅ -tyrOH			
(III)			

Our strategy, based on this computed structure, will be, for example, to determine electron transfer rates in these peptides. Removal of one deg from each tine of peptide II produces I and shortens the through-bond distance between tyrOH and trpH while negligibly changing the through-space distance. Therefore, relative to peptide II the LRET rate should be faster in peptide I if electron transfer is through-bond, but unchanged if it is through-space (Table I). Moving a deg from one tine to the other produces peptide III from peptide II and increases the through-space distance while keeping the through-bond distance fixed. Therefore, relative to peptide II, the LRET rate should be essentially unaffected in peptide III if LRET is through-bond but should be slower if it is through-space.

We have synthesized deg with a published procedure and made its t-BOC derivative in good yields with di-t-butyldicarbonate. The amino acid acc is commercially available. We have also used the t-BOCdeg in the synthesis of trpH-(deg)-tyrOH on a Biosearch SAM 2 using standard DCC coupling. Though obtaining the tripeptide trpH-deg-tyrOH by this method, we have been unsuccessful in synthesizing peptides with more than one. Coupling a deg to a tyrOH or trpH occurs readily; coupling a tyrOH or trpH to a deg occurs incompletely; we have been unable to couple a deg to a second deg, perhaps because of steric hindrance. We therefore changed our synthetic approach. Hardy and Lingham have synthesized tri- and higher homoligomers of dipropylglycine. We have adapted their synthetic scheme to make block deg peptides of length 2-5, which we then link into the final trpH/tyrOH containing peptides by either solid phase or solution methods. These deg peptides were found to be very soluble in water.

One possible problem with measuring LRET in this hairpin peptide may be its poor solubility in water, our solvent of choice. However, our energy minimized structure suggests that all the deg peptide bonds will be free to form H-bonds with the water. Thus, these peptides may be sufficiently soluble (500 mM is roughly the maximum concentration required in our peptide studies) for experiments in aqueous solutions. If the hairpins are not sufficiently soluble, then we shall switch our main effort to flash photolysis using organic solvents (e.g., acetonitrile) and to pulse

radiolysis using water/organic solvent mixtures. A second concern is whether the energy minimized hairpin is the actual solution structure. Because of the bulky ethyl side chains, it is unlikely that the oligodeg units will be anything but extended. But does the central $-(acc)_3-$ block form a b-turn? CD will be of little use, since only the tyrOH and trpH have chiral centers. However, it should be possible with 2-D NMR to identify the hydrogen bond network required of such a turn. We have access at Ohio State University to a 500 Mhz NMR that is under the supervision of Dr. Charles Cottrell, a staff chemist who has used the various techniques required for 2-D structural analysis and who is currently studying the solution conformation of our proline spacer containing peptides. If we cannot obtain sufficiently informative NMR spectra we shall turn to a less conclusive experiment. We shall seek the crystal structure of one of the three hairpin peptides. Professor Muttaiya Sundaralingam has expressed an interest in the peptides we are synthesizing, and his group at OSU is currently working with our synthetic GCN4 analog:

Ac-ala-val-lys-gln-leu-glu-asp-lys-val-**trpH**-glu-leu-leu-asn-lys-asn-**tyrOH**-lys-leu-glu-asn-glu-val-ala-lys-eu-lys-lys-leu-val-ala-glu-ala.

Our final concern is with possible large changes in the rate constants on going both from peptide II to peptide I and from peptide II to peptide III. This result is possible were both through-bond and through-space LRET occurring; but a firm conclusion might then be problematic. As an aside, this hairpin structure suggests an experiment that may test the currently popular notion that a H-bond facilitates LRET. The three peptides shown above have H-bonds only at the $-(acc)_3-$ turn. If LRET in this system were indeed through-bond, then we could arrange to "short-circuit" the transfer path by replacing one deg residue on each of the tines with isoleucine. This isomer of deg can participate in amide H-bonding and according to the Chou-Fasman rules is a good b-sheet former.

(b) OBJECTIVE 2: Does the α -Helix Affect LRET?

Our preliminary results suggest that the α -helix of our dimeric GCN4 leucine zipper analog may slow down the LRET rate, and that the Baldwin type monomeric α -helix:

Ac-ala-glu-aib-ala-ala-lys-glu-aib-ala-**trpH**-lys-glu-aib-ala-ala-lys-**tyrOH**-NH₂

may or may not affect the LRET rate. Keeping in mind that there are ambiguities with the interpretation of these data, there is at least one consistent interpretation. The tyrOH and trpH residues are strictly separated in the leucine zipper dimer, but can come into contact after the dimer melts into the monomeric random coil. Thus, melting appears to speed up electron transfer. On the other hand, melting has no major effect on the electron transfer rate in the Baldwin-type peptide, since electron transfer in this helical structure proceeds via a fast initial helix-coil transition followed by trpH/tyrOH contact. This would be possible with the Baldwin-type peptides since they are only 60-70% helical at 1 °C, and the enthalpy of the helix-coil transition is ca 13-21 Kj/mol, 4 Kj/mol lower than the observed LRET activation energy. In either case the α -helix would provide no advantage to the LRET process. However, there are important uncertainties with this type of interpretation which suggest that electron

transfer is not long range in these peptides. For example, in our preliminary study on the distance dependence of the electron transfer rate in the Baldwin-type peptide we find an apparent exponential decline with an increasing number of residues separating the two aromatic amino acids. An exponential dependence is consistent with LRET tunnelling. The leucine zipper peptide is a coiled-coil dimer of α -helices; the Baldwin-type peptide a monomeric α -helix. Thus, the two sets of data may differ only because of structural differences, with LRET inhibited in the dimeric but unaffected in the monomeric helix.

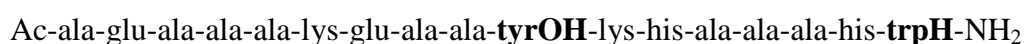
Were we able to synthesize a peptide that forms a more stable monomeric α -helix, then we might be able to verify or eliminate the possibility that electron transfer in the Baldwin-type peptide occurs primarily upon melting. Ghadri and Choi have stabilized the Baldwin-type peptide helix by binding a metal to two properly positioned histidines inserted near the peptide's carboxyl end. They obtained monomers with $> 90\%$ helicity near 0°C with metal ion addition. We propose, therefore, to synthesize an appropriate modification of these peptides; for example:



The first of these is a direct adaptation of the Ghadri-Choi peptides, the second a modification based on more recent α -helical peptides synthesized in Baldwin's laboratory. Ghadri and Choi reported that Cd^{2+} , Ni^{2+} or other transition metals stabilize the α -helical structure of their peptides. There is good reason to believe that neither Cd^{2+} nor Ni^{2+} will interfere in flash photolysis experiments since they absorb very little at 266 nm. This is also true in pulse radiolysis experiments since neither are reported to be oxidized by the azide radical, the oxidant we use to initiate LRET. (The hydroxyl radical, a more potent oxidant, cannot oxidize Cd^{2+} although it does react with Ni^{2+} .) We propose to investigate the effect of both temperature and metal concentration on LRET in such peptides.

In order to insure that helix melting cannot precede electron transfer in this metal complexed peptide, the enthalpy of the helix-to-coil transition must be larger than the ca 20 KJ/mol activation energies we have found for electron transfer in other peptides. No dependence of helical structure on temperature has yet been reported, and this will be our first experiment. If unfolding enthalpy is too low, then we can place a second -his-(ala)₃-his- unit to the N-terminal side of tyrOH to effect greater helix stabilization. (The α -helix should not be so stable that we cannot melt it at reasonable temperatures.) We have successfully synthesized peptides larger than 20 residues in the past, and we foresee no problems for the future.

These histidine containing peptides suggest an additional question; whether an intervening complexed metal ion might facilitate LRET. An approach to this problem is the comparison of LRET in the above peptides against that observed in:



In these two peptides the metal stabilizing unit lies between the tyrOH and trpH. The

possibility of electron transfer facilitation by the metal ion can be further pursued by complexing a number of different metals between the two histidines. However, Chou-Fasman rules classify tyrOH as a helix destabilizer. For this reason, the proposed peptides may not form sufficiently stable helices. (TyrOH is in either the C-terminal or N-terminal position in all other peptides already used or proposed for future use. We could presumably do so in this case as well. However, our data cited above suggest that ordering of the two amino acids in the peptide chain may of itself affect the LRET rate). We shall, however, know this only after at least one of these peptides has been synthesized and tested. With everyone of these helical peptides we shall utilize the same experimental protocol described earlier. Namely, a peptide is expected to be helical at low temperature and melt at higher temperatures. The LRET rate constant will be measured as a function of temperature with the expectation that an effect of the helix on the LRET rate would be seen either as a markedly nonlinear Arrhenius plot or as an activation energy characteristic of helix unfolding and not of electron transfer. The helicity of every peptide will be checked with CD, and we shall perform a full 2-D NMR analysis with at least one peptide of each type.

(c) **OBJECTIVE 3: How Does a Fully Extended Peptide Chain Affect LRET?**

LRET occurs in systems other than peptides and proteins. Among these is the very rapid electron transfer between donor and acceptor separated by a fully saturated norbornyl-type ring system. Hush has suggested that the regular, fully extended bonding progression through the spacer in this system may support the very rapid electron transfers. In contrast, oligoproline forms an elongated spiral that resembles the collagen helix. The oligodeg spacer should be fully extended as suggested by the x-ray crystal structures of both deg and dipropylglycine derivatives. In line with Hush's suggestion, it will be interesting to see if the LRET rate across the $-(\text{deg})_n-$ spacer is much different than that across $-(\text{pro})_n-$. We also suggest to use hydroxymethylserine (hms) as the spacer since it is at least as hindered as deg and so should also prefer the extended conformation. There is no literature report for the large scale synthesis of an hms derivative suitably protected for subsequent peptide chemistry. So we have synthesized the dibenzyl-hms from the dichloro alcohol with an overall yield of 60-70%. With the dibenzyl derivative we have already synthesized the series $\text{trpH}-(\text{hms})_n-\text{tyrOH}$ and $\text{tyrOH}-(\text{hms})_n-\text{trpH}$. Here again we shall perform a full 2-D NMR analysis with at least one of each type of these peptides.

(d) **OBJECTIVE 4: How Do Inter- and Intra-chain Electron Transfer Compare?**

So far we have dealt only with LRET within a polypeptide chain. However, Hoffman et al. have reported LRET between the α - and β - subunits of a mixed metal [Zn(II),Fe(III)] heme hybrid hemoglobin, and McLendon et al. have described electron transfer between the two proteins in the Cytochrome b_5 /Cytochrome c complex. Therefore, LRET can occur between polypeptide chains held together in a complex. In order to model such an interchain LRET, we cannot just tie two peptides together with a disulfide bond. The disulfide itself can be oxidized (or reduced) to an unstable radical cation (or anion), thereby potentially confusing any study of LRET between the two chains. There is, however, a peptide system that may be appropriate for these studies; O'Shea, et al. (90) have shown that two peptides

based on the *fos* and *jun* nuclear oncogene products form a-helical parallel heterodimers. With these as models (analogs of these two peptide fragments have already been synthesized in Kim's laboratory, personal communication), we propose to synthesize pairs of peptides for the comparison of inter- and intra-chain electron transfers. In one pair tyrosine and tryptophan will be incorporated into the same chain, in the other they will be located at similar positions but separated onto the complementary chains. Experimental manipulation will come by moving the two amino acids with respect to one another on the chains. Structural melting (on raising the temperature) results in an interesting control here. With tyrOH and trpH on the same chain, unfolding should give the same results we obtain with the GCN4 dimer. With tyrOH and trpH on different chains, we would expect the rate constant to drop to zero as the peptide chains dissociate, for under the conditions of our experiments intermolecular electron transfer is negligible. This proposed dimer system should also be useful in another context. As previously described, between the Baldwin-type and the "GCN4" homodimer helices there is an apparent difference in the dependence of the LRET rate on temperature. An important structural difference between these two peptide systems is that the homodimer contains two tyrosine/tryptophan pairs in one molecular complex, one pair on each chain. Since the homodimer associates in a parallel manner, one might not expect an important effect due simply to the presence of two redox center pairs. This expectation can be tested with a heterodimer that has a tyrosine/tryptophan pair on only one of the two strands.

(e) **OBJECTIVE 5: What is the Effect of the Equilibrium Driving Force (ΔG^0) on LRET Rates?**

While there is evidence that the dependence of LRET rates on ΔG^0 may be consistent with Marcus theory within one class of proteins, this has yet to be shown in peptides. Moreover, the apparent directional specificity of the tyrOH/trpH system is not consistent with Marcus theory. Therefore, we propose to explore the effect of ΔG^0 on both LRET rates and the apparent directional specificity. We shall start with a peptide in which ΔG^0 , the LRET equilibrium driving force, is approximately 0 Volt. We know that the redox potential of the radical formed in the 1-electron oxidation of the tryptophan derivative tryptoline-3-carboxylic acid, [3S]-1,2,3,4-tetrahydro-carboline-3-carboxylic acid (tlcH), is lower than that formed by trpH by 50 mV. Since the potential of the trp[•]/trpH couple is roughly 50 mV greater than that of tyrO[•]/tyrOH in the peptides tyrOH-(pro)_n-trpH, we would expect an approximate ΔG^0 of zero between tyrOH and tlcH in the peptide tyrOH-(pro)_n-tlcH. While not a major change in ΔG^0 , we are interested in seeing how a system with essentially no equilibrium driving force sustains an LRET. TlcH is easily synthesized, and we have already made t-BOC-tlcH for use in standard peptide synthesis.

For systems with larger ΔG^0 , we propose to investigate first the 1-electron oxidation of ferrocene (Fc) by trp[•], where the redox potential difference will be in the range of 0.3 to 0.4 V. We have already shown a rapid trp[•] oxidation of monocarboxylic-ferrocene (Fc-COO⁻) and important for establishing a subsequent net electron transfer, the rates of tryptophan and Fc-COO⁻ oxidation by N₃[•] and the extinction coefficients of tryptophan and Fc-COO⁻ at 266 nm are roughly the same. While we have already attached Fc-COOH as an amide to the trpH, Fc-CO- cannot be installed at the C- terminal end of the peptide, as would be required for looking at

directional specificity by interchanging Fc and trpH positions. We, therefore, propose the synthesis of the ferrocenyl amino acid (FcA). Synthesis, with no racemization, of t-BOC-L-serine -lactone from t-BOC-L-serine has been reported. Nucleophilic attack on the serine with ring opening occurs readily, and there is precedent for the Grignard attack at the methyl position with copper as catalyst. The ferrocenyl Grignard can be made from commercially available hydroxymethyl ferrocene. If we can successfully:

- i) synthesize peptides with a ferrocenyl side chain and
- ii) show LRET to the tryptophan radical,

then we can explore the reactions of other amino acid metallocenes. This will permit us the wide range of redox potential differences to verify the Marcus theory in peptides.

(f) **OBJECTIVE 6: What is the Effect of the Solvent on LRET Rates?**

There is evidence that the dependence of LRET on λ_{out} may be consistent with Marcus theory within one class of proteins. This has yet to be shown in peptides. Most of the work on peptides was limited to aqueous solutions and this important parameter was never investigated. Therefore, we propose to explore the solvent effect on LRET using 266 nm photons. We shall start our flash photolysis studies with the trpH-(pro)_n-tyrOH and tyrOH-(pro)_n-trpH peptide series which we have studied in aqueous solution by pulse radiolysis. Preliminary investigations have shown that these peptides are soluble in alcohols (methanol, ethanol, 1-propanol and 1-butanol) nitriles (aceto-, propio- and benzo-), and dimethylsulfoxide with λ_{out} ranging from 0.95 to 0.62 eV, and therefore suitable for this investigation.

The magnitude of λ_{out} is generally calculated from a classical model (98). In all cases λ_{out} increases with increasing separation of the redox couples. Also the consequence of changes in ΔG^0 (objective 5) and λ_{out} depends upon whether the LRET reaction is in the normal or in the inverted region and can be quite complex. In the normal region increasing λ_{out} decreases the rate; however, in the inverted region, increasing λ_{out} increases the rate.

(g) **OBJECTIVE 7: Triplet-Triplet Energy Transfer and LRET**

One of the most interesting result obtained in our previous studies with the proline peptides was the value for b (0.3 - 0.4), which is significantly smaller than the estimated theoretical value of 1.2 - 1.5. Such discrepancies have dramatic consequences for predicted reaction rates (several orders of magnitude). Therefore, an independent technique for the determination of b is required to check these findings. We shall to determine b from the rate constants of the triplet-triplet energy transfer. Since it is well known that 266 nm photons produce tryptophan triplet we shall start our study of triplet-triplet energy-transfer with the trpH-(pro)_n-tyrOH and tyrOH-(pro)_n-trpH. There are two processes that we have to eliminate before implying a genuine intramolecular triplet-triplet energy transfer, namely:

- i) an intermolecular process (independence of concentration); and
- ii) a two-photon process (allowed triplets).

For this we need a powerful laser system to be able to vary the excitation power by at least 10- fold. In this respect our Sherbrooke system meets these requirements. Moreover, the possibility to investigate processes in the sub-picosecond region is most interesting.

7.4 ELECTRON AND PROTON TRANSFER IN FLAVOPROTEINS.

This project is concerned with the application of pulse radiolysis to two problems in protein redox reactions: to delineate those fast processes which accompany the one electron reduction of flavoproteins and to obtain a more definitive picture of intramolecular electron and proton migration within an intact protein. This project is a continuation of our previous studies with Flavodoxin (109,113) and riboflavin binding protein (128,137,138).

The search for electron and proton migration within proteins is a relatively new concern to protein chemists. There have been evidences for such intramolecular migration, and we hope that with this study we could delineate more clearly these still vaguely understood processes. Intramolecular electron and proton movement may be important in the mechanism(s) of physiological redox reactions which involve proteins either as redox catalysts or electron carriers. But there is another, unique factor to these experiments. We have accumulated evidences (106,107,110,136) that e_{aq}^- shows an apparent reductive specificity for the active site. It is generally accepted that the distribution of charged groups on a protein's surface affects the interaction of that protein as big and small molecules. By extension, the non-uniform distribution of both charged and dipolar groups on and within a low dielectric protein interior surely results in a "potential energy surface" which plays an important role in establishing the biological properties of proteins. If an electron or a proton migrate within the protein and can be influenced by such a potential surface, then, the apparent anomalous specificity of the electron or the proton for the active site is the reflection of a fundamental enzyme property, knowledge of which would be important to our understanding of enzyme catalysis.

Although not extensively utilized with flavoproteins until now, the technique of pulse radiolysis is particularly well suited to the analysis of electron and proton transfer in flavoproteins. Radical reductants such as e_{aq}^- , CO_2^- and carbon centered radicals or the more specific NAD and RSSR⁻ radicals can be introduced into the aqueous protein solutions in less than a microsecond. Because NAD and RSSR⁻ absorb strongly in the visible, the products of the various possible reactions on flavoproteins are readily monitored. Additionally, those pulse radiolysis results that have been previously published indicate that intermediate reaction steps are too fast to be detected by more traditional techniques, such as stopped flow. The problem we propose to pursue may have some relevance to the mechanism of flavoprotein catalysis and may lead to some understanding of how the various flavoproteins control the pK of their flavin group.

Finally, we would like to note that the project described herein is related by the common teams of intramolecular proton and electron migration in proteins. Both processes are of importance in understanding those enzyme reaction mechanisms postulated to include proton transfer steps, and protein redox reactions. We have with the pulse radiolysis method been able to observe both these processes. Few other

methods have the necessary speed to permit such observations. Perturbation experiments such as T-jump, which allows to make measurements in this same time scale, require an equilibrium or a steady state system. With pulse radiolysis we can survey the entire time course of a reaction, and hence easily follow functionally irreversible reactions. Our goal is to continue uncovering experimental evidences for these two classes of intramolecular reactions in order to describe their properties and ultimately reveal their mechanism.

We propose to study the pulse radiolysis of aqueous Flavodoxin solutions with three goals in mind. First we wish to delineate intramolecular electron migration in the protein after an initial reduction of the protein by the above mentioned radicals. Second, we shall attempt to find electron transfer between flavin and disulfide groups, a transfer which may be an integral in the mechanism of this enzyme. Finally, we shall explore the kinetic consequences of the protein structural control on the pK of the N-5 site in the flavin semiquinone.

7.5. ELECTRON AND PROTON TRANSFER REACTIONS IN DEHYDROGENASES AND THE CHEMISTRY OF THEIR COFACTOR METHOXATIN

Methylotropic bacteria are distinguished by their ability to utilize single carbon compounds such as methanol and methylamine as the sole source of carbon and metabolic energy. These bacteria have been found to contain dehydrogenases which require a novel anthraquinone cofactor called Methoxatine rather than the more commonly required nicotinamide and flavin cofactors.

It has been demonstrated that these dehydrogenases are able to stabilize the semiquinone form of Methoxatine. In several cases the protein stabilized semiquinone can be produced merely by adding substrate to the fully oxidized enzyme and in the absence of a terminal electron acceptor. These observations suggest the involvement of the semiquinone in the catalytic mechanism of Methoxatine dependent dehydrogenases. Until recently little work has been done concerning the electron transfer mechanism of the reduction of these dehydrogenases and the physical and chemical properties of their cofactor. Interest in this novel cofactor had increased with recent discovery of a Methoxatine dependent mammalian amine oxidase.

Recently, we have done a preliminary study on the chemical properties of the methoxatin at physiological pH (142). Rate constants for the reduction of the cofactor by CO_2^- and O_2^- , the transient spectrum of the semiquinone, the redox potential of the quinone/semiquinone couple and the bimolecular decomposition of the Methoxatine semiquinone were determined.

Further investigations on the chemical properties of Methoxatine are suggested. These include the Ph effect on the above properties, electron transfer reactions from and to the Methoxatine (in both oxidation states) and an attempt to stabilize artificially the semiquinone by binding it to host protein. We would also like to start a systematic investigation on the enzyme. The guide lines for this investigation will be similar to those outlined in our previous publications (109,128,137,138) and in the previous section of this chapter on the electron and proton transfer reactions in flavodoxin. The first question we would like to investigate is: does protonation of the semiquinone in the protein is an essential step in the stabilization of the protein.

7.6. ELECTRON TRANSFER REACTIONS IN DNA STRUCTURAL UNITS.

Free radical reactions have been implicated increasingly not only in the damaging effects of radiation on living organisms but also in the initiation and progression of various diseases and in the toxic action of numerous drugs and chemicals. It appears that the living cells are continuously opened to the attack of free radicals which damage mainly the DNA (by strand breaking) and membranes contributing largely to the cell killing. In aqueous solutions strand(s) breaking results mainly from oxidation reactions (e.g. OH radicals) with a final alteration of the deoxyribose. OH radicals react rapidly with purine and pyrimidine bases ($6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and less effectively with the sugar ($1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$). In nucleosides more than 80% of the OH radicals react with the base as judged from the rate constants and the transient spectra resulting from the radical reaction. Pulse radiolysis studies with DNA showed that the OH rate constant is one order of magnitude slower with the DNA. The transient spectrum was much more complicated with some similarities to the spectra found for the nucleotides. Single strand break (SSB) in DNA results from a radical formation in position C(4) of the sugar, if only the reaction at this position of the sugar with OH radicals was taken into consideration the yield (G value) of SSB should have been 0.24 (based on competition between the C(4) hydrogen and all other sites capable to react with OH). The experimental results for this rupture are much higher and recent studies claim to attain a yield of 2.4 (Schulte-Frohlinde, personal communication). The implication of this number is that one of each two OH radicals results in SSB. Consequently we have to assume that internal H atom transfer (or electron followed by proton transfers) from the base to the sugar is occurring.

We propose to extend and expand the study of the oxidation of DNA and its constituents. We plan to investigate the reaction of OH, Br_2^- , $(\text{CNS})_2^-$ and N_3 radicals and to look into the factors affecting their rate constants. Among these factors we plan to test: (a) the chain length of the biomolecule; (b) charge effect; and, (c) inductive effects. We shall investigate the resulting transient absorption spectra with respect to the pH, ionic strength and the species constituting the biomolecule and its concentration. From these results we expect to postulate the mechanism and calculate the yield of the different radicals formed during the initial phase(s) in the radiolysis of DNA. The possibility of internal hydrogen transfer from the sugar moiety to the attacked base, a mechanism by which chain breaks yield may increase will be also tested. This study will be performed along the guide lines described in reference 129.

In the second phase of this research project, an extension of the experiments described in section 7.6 but with bases, nucleosides and nucleotides as spacers will be performed. These experiments will allow us to investigate not only the electron transfer process along a helical spacer but more important result may lay in the effect of the presence of the sugar and phosphate on this process.

7.7. THE CO_3^- RADICAL ION AS AN OXIDIZING AGENT.

It is generally assumed that oxidative agents (OH radicals) are responsible for the single and double strand break in DNA. However, OH radicals are potent radicals known to react with practically any molecule present in the cell. On the other hand, it

is a known fact that bicarbonate is the most abounded compound present in a cell. This ion reacts quite effectively with OH radicals ($k = 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4) with the formation of a CO_3^- radical. This radical ion is expected to be a less powerful oxidant and therefore more specific than OH radicals. It absorbs in the visible region (band centered at 600nm) therefore, easy to follow and with relatively long half life (its natural decay is via radical-radical recombination reaction ($k = 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4)). Thus the half life of 10^{-9} M of CO_3^- in water at pH 7.4 will be 100 seconds. We suggest to study the reactions of CO_3^- with structural units of DNA at physiological pH.

7.8. SOD AS A CATALYST FOR THE DISMUTATION OF FREE RADICALS.

Since the discovery (1969) of superoxide dismutase protein (SOD) as a catalyst for the disproportionation reaction of O_2^- , one question has not been answered: why does a biological system need such a catalyst? Many studies have shown that the reactivity of O_2^- at the physiological Ph toward almost every chemical unit in the cell (proteins, DNA, membranes) is very low and therefore not harmful to the biological function of the cell. Other possibilities were proposed one of which tries to link the O_2^- with the formation of OH radicals or "OH like" radicals. These radicals are produced via the reaction of the superoxide radical with hydrogen peroxide and is catalyzed by metal ions. If this is the case then, as previously noted, the attack will be highly un-specific with the formation of secondary radicals, as suggested in section 7.8. The question of SOD being a specific catalyst for the superoxide radical was raised the moment it was discovered. One possibility is that the protein is in fact a good complex formation unit which produces a metal-radical complex intermediate and that the complex does formed reacts with another radical in a fast reaction. Thus SOD will catalyze any radical-radical dismutation reaction independent of their nature (oxidizing or reducing). The fact that metal ions are associated with the active site in SOD and posses different oxidation states is favoring such a proposal.

It is the object of this project to investigate the catalytic activity of SOD towards the dismutation reaction of some secondary radicals produced during the radiolysis of aqueous solutions.

7.9. PHOTOLYSIS OF PHTALOCYANINES IN SOLUTION. A SPIN TRAPPING AND E.S.R. STUDIES.

It has been known for some time that porphyrin and porphine compounds (in the presence or absence of metal ions) can be used as photosensitizers to induce radical reactions in biological systems. Different radicals have been found during these photoreactions. These were OH radicals, hydrated electrons, superoxides and singlet oxygen. The type of radical depending on the nature of the macrocyclic compound.

During the last two decades an increasingly interest in properties of hematoporphyrins and its derivative(s) (HPD) was shown. HPD was used first as a diagnostic tool to differentiate between malignant and normal tissue (it is preferentially accumulated in malignant tumors) and later was found to be useful in the phototherapy of tumors. The problem with the use of these compounds is their

extraordinarily large absorption in the 400nm region which makes its medical application very delicate. Very recently, phtalocyanines were found to exhibit properties similar (or better) to those found with HPD. These compounds have the advantage that their main absorption band is in the red with an extremely high extinction coefficient. Therefore these compounds seem more suitable for tumor treatment using laser photoactivation.

We would like to study, as we have done with water soluble metalloporphyrins; solution properties and ligand binding in metal containing phtalocyanine(s), the reaction of these compounds with pulse radiolytically formed radicals, and the nature of radicals produced during the photolysis of phtalocyanines. Sulfonated and positively charged phtalocyanines in aqueous solutions will be used.

These properties will be compared with commercial phtalocyanines in non aqueous solutions. The binding of different ligands (small inorganic, amino acids, peptides, proteins and nucleic acid bases) will be carried on with special interest involving the problem of how these compounds are moving into the cell. We suspect that phtalocyanines are associated with one of the biochemical ligands enabling them to cross the cell membrane(s). In addition to these experiments electrochemical studies on the redox potential of various metal containing phtalocyanines in aqueous and non-aqueous solutions will be investigated.

8. RESEARCH SUPPORT

- 8.1 Israeli National council for Research- #3561-1-86 -1/9/86 - 31/8/89
Moshe Faraggi, Principal Investigator - 20% time
Total Amount: \$80,000
1st Year Amount \$26,500
"Long Range Intramolecular Electron Transfer Reactions in α - Helix Polypeptides"
- 8.2 U.S.-Israel Binational Science Foundation - BSF - 85-00217 - 1/9/86 - 31/8/89
Moshe Faraggi, Principal Investigator - 20% time
Michael H. Klapper, Collaborating Scientist, O.S.U
Total Amount: \$90,000
1st Year Amount \$25,500
"Long Range Intramolecular Electron Transfer Reactions in α - Hemoglobin"
- 8.3. National Institute of Health U.S.A., NIH - 1 RO1 GM35718-01A1 - 1/7/86 - 30/6/91
Michael H. Klapper, Principal Investigator - O.S.U.
Moshe Faraggi, Collaborating Scientist - 25% time
Total Amount: \$536,567
1st Year Amount: \$99,167
"Intramolecular Electron Migration in Proteins and Polypeptides"
- 8.4. U.S.-Israel Binational Science Foundation, BSF - 86-00206 - 1/9/87 - 30/8/90
Moshe Faraggi, Principal Investigator - 20% time
Michael H. Klapper, Collaborating Scientist O.S.U.

Total amount: \$100,000

1st Year Amount: \$30,000

"Intramolecular Electron Transfer Across Polypeptide Spacers"

8.5. U.S.-Israel Binational Science Foundation, BSF - 90-00249 - 1/9/91 - 30/8/94

Moshe Faraggi, Principal Investigator - 20% time

Michael H. Klapper, Collaborating Scientist, O.S.U.

Total amount: \$139,450

1st Year Amount: \$34,000

"Intramolecular Electron Transfer Across Polypeptide Spacers"

8.6. Arc-en-Ciel, France-Israel Science Foundation

Chantal Houee-Levin, French Principal Investigator - 15% time

Moshe Faraggi, Israeli Principal Investigator - 15% time

Exchange visitors program.