Haest, C. W. M., & Deuticke, B. (1976) *Biochim. Biophys. Acta 436*, 353-365.
Haest, C. W. M., Plasa, G., Kamp, D., & Deuticke, B. (1978) *Biochim. Biophys. Acta 509*, 21-32.
Kimmich, G. A., Randles, A. J., & Brand, J. S. (1975) *Anal. Biochem. 69*, 187-206.
Kobayashi, T., Okamoto, H., Yamada, J.-I., Setaka, M., & Kwan, T. (1984) *Biochim. Biophys. Acta 778*, 210-218.
Laggner, P., Gotto, A. M., & Morrisett, J. D. (1979) *Biochemistry 18*, 164-171.
Lange, Y., & Slayton, J. M. (1982) *J. Lipid Res. 23*, 1121-1127.
Lew, V. L. (1971) *Biochim. Biophys. Acta 233*, 827-830.
Lieber, M. R., Lange, Y., Weinstein, R. S., & Steck, T. L. (1984) *J. Biol. Chem. 259*, 9225-9234.
Mohandas, N., & Feo, C. (1975) *Blood Cells 1*, 375-384.
Mohandas, N., Greenquist, A. C., & Shohet, S. B. (1978) *J. Cell. Biochem. 9*, 453-458.
Mohandas, N., Wyatt, J., Mel, S. F., Rossi, M. E., & Shohet, S. B. (1982) *J. Biol. Chem. 257*, 6537-6543.
Mombers, C., Verkleij, A. J., DeGier, J., & Van Deenen, L. L. M. (1979) *Biochim. Biophys. Acta 551*, 271-281.
Nakao, M., Nakao, T., & Yamazoe, S. (1960) *Nature (London) 187*, 945-946.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47-71.
Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579-598.
Pjura, W. J., Kleinfeld, A. M., & Karnovsky, M. J. (1984) *Biochemistry 23*, 2039-2043.
Reed, P. W., & Lardy, H. A. (1972) *J. Biol. Chem. 247*, 6970-6977.
Roelofsen, B., & Zwaal, R. F. A. (1976) *Methods Membr. Biol. 7*, 147-177.
Rosenberg, R., Young, J. D., & Ellory, J. C. (1980) *Biochim. Biophys. Acta 598*, 375-384.
Schrier, S. L., & Junga, I. (1981) *Proc. Soc. Exp. Biol. Med. 168*, 159-167.
Schwartz, R. S., Chiu, D. T.-Y., & Lubin, B. (1985) *Curr. Top. Hematol. 5*, 63-112.
Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751-3755.
Sheetz, M. P., & Singer, S. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4457-4461.
Sweet, C., & Zull, J. E. (1970) *Biochem. Biophys. Res. Commun. 41*, 135-141.
Tanaka, Y., & Schroit, A. J. (1983) *J. Biol. Chem. 258*, 11335-11343.
Tsien, R. Y. (1981) *Nature (London) 290*, 527-528.
Tsien, R. Y., Pozzan, T., & Rink, T. J. (1982) *J. Cell Biol. 94*, 325-334.
Van Deenen, L. L. M. (1981) *FEBS Lett. 123*, 3-15.
Van Meer, G., & Op den Kamp, J. A. F. (1982) *J. Cell. Biochem. 19*, 193-204.
Young, J. D., & Ellory, J. C. (1977) in *Membrane Transport in Red Cells* (Ellory, J. C., & Lew, V. L., Eds.) pp 301-325, Academic Press, New York.
Zlatkis, A., Zak, B., & Boyle, A. J. (1953) *J. Lab. Clin. Med. 41*, 486-492.

Reactivities of Tyrosine, Histidine, Tryptophan, and Methionine in Radical Pair Formation in Flavin Triplet Induced Protein Nuclear Magnetic Polarization

Karol A. Muszkat* and Tmima Wismontski-Knittel

Department of Structural Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Received September 19, 1984; Revised Manuscript Received March 7, 1985

ABSTRACT: In order to check the validity of several basic assumptions of protein photochemically induced nuclear polarization (protein photo-CIDNP), we have investigated the quenching processes of the dye triplets by the side chains of tyrosine, histidine, and tryptophan in a variety of molecular systems and environments. The quenching (H atom or electron transfer) is the generating process of the triplet electron-spin-correlated radical pair, the evolution of which gives rise to nuclear polarization. At pH 7 the quenching of 10-(carboxyethyl)flavin triplets by tyrosine and tryptophan is almost diffusion controlled. Quenching by histidine is slower. We have also investigated the slow quenching (by electron transfer) by the side chains of methionine and could show that quenching by cysteine S derivatives is negligible. Quenching by tyrosine and histidine peptides and by the tyrosines of the pancreatic trypsin inhibitor protein is slightly slower than by free side chains. Quenching is strongly viscosity controlled, to be expected of a process requiring bimolecular contact. Reactivity trends at high viscosities resemble those observed in fluid aqueous solutions. Activation energies of quenching by tyrosine, tryptophan, and histidine are similar. No difference could be detected in the mechanism of quenching by these side chains. No fast static quenching was observed that could compete with the diffusional process.

Protein photo-CIDNP[1] measurements serve as a highly specific and sensitive tool for studies of accessibility of active[1] side chains of proteins in solution. The same measurements show promise in studies of protein-protein interactions, as a

0006-2960/85/0424-5416$01.50/0

source of detailed information on accessibility changes associated with a variety of processes such as conformational transitions, aggregation, and enzyme–inhibitor and enzyme–substrate binding, as well as general protein–protein interactions [see, e.g., Muszkat et al. (1982, 1983, 1984a,b); K. A. Muszkat and I. Khait, unpublished results]. Accessibility of "CIDNP-active"[1] peptide side chains to the triplet flavin dye probe is a clear molecular prerequiste for the observation of protein nuclear magnetic polarization effects [for a recent discussion, see, e.g., Muszkat et al. (1984a,b)]. This situation is due to the basic requirement of genuine contact or at least very close approach of the triplet flavin (or fluorescein derivative) ^3D to the reactive atomic centers of the side chain to allow the transfer of an H atom as such (process 1)[2]

$$A\text{–}H + {}^3D \rightarrow \overline{{}^3A\cdot + DH\cdot} \qquad (1)$$

or possibly to allow the transfer of an electron, as in the related process (2)

$$B + {}^3D \rightarrow \overline{{}^3B^{+}\cdot + D^{-}\cdot} \qquad (2)$$

In protein photo-CIDNP experiments involving a given side chain, provided the rates of spin–lattice relaxation are equal, the rates of processes 1 and 2 are the decisive kinetic factors that determine the intensities of observed nuclear polarization effects. These rates control the formation of the spin-correlated radical pairs. Their values are determined by the accessibilities as well as by the inherent reactivities of completely accessible side chains of tyrosine, histidine, and tryptophan.

The observation of nuclear polarization in flavin triplet quenching by amino acids and proteins is a clear indication for fast radical pair formation in (1) and (2). The CIDNP effect by itself indicates that the rates k_q of processes such as (1) and (2) are higher than a threshold of ca. 10^6 s^{-1} (Muus et al., 1977). The few k_q values available in the literature prior to the outset of this work, e.g., for *N*-acetyltyrosine ethyl ester and for tryptophan, fully support such a contention. Thus, triplets of lumiflavin, riboflavin, and several N-10-substituted flavins are quenched by *N*-acetyltyrosine ethyl ester with rate constant values close to 2×10^9 M^{-1} s^{-1} (in water–3% dimethylformamide solution; McCormick et al., 1975; McCormick, 1977). Riboflavin triplet is quenched by tryptophan with a quenching rate constant (k_q) value of 2.5×10^9 M^{-1} s^{-1} (Heelis & Phillips, 1979), while the highest quenching rate constant reported is for lumiflavin triplet with 2,6-dimethylphenol, $k_q = 4.9 \times 10^9$ M^{-1} s^{-1} (Vaish & Tollin, 1970). These values are still below some diffusion-controlled limits of ca. 1.1×10^{10} M^{-1} s^{-1} in electron-transfer processes of transition-metal complexes (Holzwarth, 1982) and below the estimated electron-transfer limit of 10^{11} M^{-1} s^{-1} (Bruhn et al., 1982).

In this work we intend to address ourselves to several problems encountered when attempting to deduce information on protein conformation and local accessibilities of active side chains from CIDNP intensity data. The first such problem is faced whenever several different accessible residues occupy neighboring sites. In such a situation it is important to evaluate the effects of side chain competition for ^3D, as the polarization of residues possessing the largest inherent reactivity could predominate in this case due to competition in processes 1 and 2 discriminating against the appearance of polarization in the less reactive residues. The second problem concerns the possibility of competition by triplet quenching processes analogous to (1) and (2) but from residues that are unable to exhibit proton CIDNP (having no protons suitably nuclear-spin coupled by hyperfine interaction to the unpaired electron spin in $A\cdot$ or $B^{+}\cdot$). Prominent among such residues are those with thioether or disulfide groups that because of low ionization energies are prone to electron-abstraction reactions similar to (2). The third problem involves the detailed mechanism of radical pair formation, leading possibly to a different ^3D–side chain separation dependence of quenching by different side chains. Thus a hydrogen transfer such as (1) would differ from an electron transfer such as (2) in the extent of quantum mechanical tunneling involved (Bell, 1980). A process such as (2), provided that the electron is transferred free and not bound to solvent, is expected to show a larger participation of quantum mechanical tunneling than (1). However, the situation would be reversed if the hydrogen atom is transferred free while the electron is transferred tightly bound to solvent. Thus in the first case, (2) could take place at a larger separation than (1) because of tunneling, the opposite being true in the second case, though to a lesser extent.

The present-day evidence for the exact extent of reaction by each mode in each of the three active amino acid residues is indirect and somewhat incomplete. Phenols and tyrosine show larger polarization than phenoxides while O-methylated derivatives are inactive [see, e.g., Muszkat & Weinstein (1975, 1976a,b) and Muszkat et al. (1984 a,b)], leading to the conclusion that H atom transfer is largely involved. The same probably holds for histidine. In the case of tryptophan it can be shown that electron transfer operates in the 1-methyl derivative (McCord et al., 1981) where H transfer is *impossible*. Whether the same conclusion holds for tryptophan where H transfer is *possible* seems as yet unsettled.

Finally, the last problem we wish to consider is that of the extent of the static quenching path vs. the dynamic, diffusion-dependent path [cf., e.g., Birks (1970), pp 393 and 450). This problem is different from that of the determination of the role of possible dye–amino acid residue ground state complexes. The data of the equilibrium constants for flavin complexes (ca. 1 for histidine; ca. 10^2 for tyrosine and tryptophan) (Draper & Ingraham, 1970) indicate that *complexes* are unimportant as reactive components at low concentrations.

With these problems in mind we measured the rates of flavin triplet quenching by a series of accessible active residue substrates. Among these substrates we included *N*-acetyl derivatives of the amino acids and several different peptides, as well as a typical globular protein. The conditions—medium, viscosity, and temperature—were chosen so as to make evident differences in the quenching mechanisms of the various active residues. These new measurements considerably extend the scope of the previous measurements mentioned above. The method chosen for these measurements is the direct optical monitoring of the flavin triplet decay by microsecond flash photolysis. For accounts of flavin triplet processes, see, e.g., Tegner & Holmstrom (1966) and Heelis (1982); cf. also Ahmad et al. (1981).

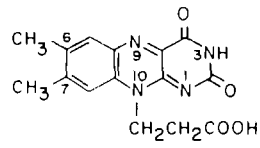
Equating the rate of the triplet decay with the rate of radical pair formation implies that other parallel deactivation processes of the triplet competing with radical pair formation are of only minor importance. Indeed, the experimental results to be shown below, taken together with the photo-CIDNP results,

[1] CIDNP is chemically induced dynamic nuclear magnetic polarization, referred to here also as nuclear polarization [see, e.g., Muus et al. (1977)]. "CIDNP-active" side chains which exhibit protein photo-CIDNP effects are those of tyrosine, histidine, and tryptophan [see, e.g., Muszkat & Gilon (1978), Müller et al. (1980), and Muszkat et al. (1984)], as well as suitably modified side chains of lysine (Lerman & Cohn, 1980) and of cysteine (Jacobson et al., 1983).

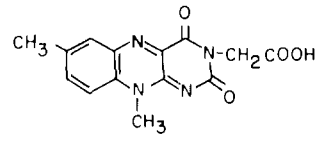
[2] Electron-spin correlation is indicated by a horizontal bar. The left superscript indicates the total electron spin state. The left-hand-side reaction of processes 1 and 2 is known also as triplet quenching.

seem to justify this assumption.

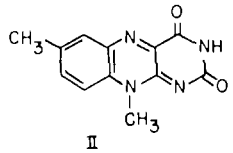
The choice of the optimal triplet dye probe for protein CIDNP studies is dictated first of all by considerations of sufficient inherent triplet reactivity to provide for efficient radical pair formation. This consideration has to be supplemented by the practical requirement of stability toward irreversible photochemical fading. For this last reason the dye selected for our recent photo-CIDNP studies [see, e.g., Muszkat et al. (1982, 1983, 1984a,b)] has been 10-(carboxyethyl)flavin (I). This dye was first introduced for the photo-CIDNP study of pyridoxal-derivatized lysines of proteins (Lerman & Cohn, 1980).



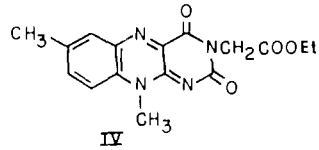
I



III



II



IV

Most of our efforts in this study were therefore devoted to the detailed investigation of the reactivity of the triplet of this flavin derivative. However, for comparison on some points we have also examined other flavin derivatives, e.g., 6,10-dimethylisoalloxazine (II), 3-(carboxymethyl)-6,10-dimethylisoalloxazine (III) and its ethyl ester (IV), 10-phenylflavin, 10-(carboxymethyl)flavin, riboflavin, and flavin mononucleotide (FMN).

Materials and Methods

10-(Carboxyethyl)flavin (Föry et al., 1968) was kindly provided by Dr. D. Porter (Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA). 6,10-Dimethylisoalloxazine and the *N*-acetyl derivatives of tyrosine, tryptophan, histidine, and methionine, as well as reduced and S-carboxymethylated glutathione, were all provided by Dr. S. Weinstein (The Weizmann Institute of Science, Rehovot). Tyr-Glu, Tyr-Tyr, and Tyr-Tyr-Tyr were products of Miles-Yeda Ltd., Rehovot, while the other peptides used were kindly provided by Prof. M. Wilchek (The Weizmann Institute of Science, Rehovot). The sample of S–S glutathione (oxidized) was obtained from Sigma Chemical Co., St. Louis, MO. The sample of bovine pancreatic trypsin inhibitor (BPTI, Trasylol) used was the gift of Bayer AG, Werk Elberfeld. The ethylene glycol used was a pure grade, 99% by gas chromatography (GC), supplied by Merck Co. (Darmstadt).

A conventional design microsecond flash photolysis setup was used for the flavin triplet decay studies. The flash unit consists of two air-filled capillary flash tubes (15-mmHg pressure, 10-cm interelectrode distance) energized by one or two 200-J capacitors. The flash profile is 12 μs wide at half-intensity. Measurement of transient decay is started 35 μs after the beginning of the discharge. The flash light is filtered with a blue glass filter having a transmission maximum at 450 nm (Corning Co. no. 5031). The analytical light source is a 50-W tungsten–iodine projection lamp. This light is filtered with a 2-cm-thick water layer. The sample solution is contained in long-necked cells of 2-cm path length. The cell thickness (exciting-light path length) is 0.4 cm, and its height is 1.5 cm. Red transmitting glass filters (Schott and Co. no. RG610) were inserted at the entrance and exit of the sample cell compartments. The photometric unit consists of a 500-mm focal length grating monochromator (Bausch and Lomb) and a red-sensitive photomultiplier (EMI 9558A). The overall dynode voltage is 750 V. The signal is measured with a digital oscilloscope (Nicolet Co. Model Explorer III). Measurements were carried out at the flavin triplet absorption maximum at 710 nm (680 nm in acid solution). Oxygen was removed by bubbling a slow stream of pure argon for 30 min. Viscosity was varied by changing the temperature of sucrose solutions [30–67% by volume; for viscosity–temperature data, see Barber (1966)] and of a 70% ethylene glycol–water mixture [for viscosity vs. temperature data, see Eastman & Rosa (1968)]. The viscosities of the various media used are indicated in Table I and Table II. The second-order quenching rate constants k_q are related to the first-order decay rate constants k_{exp} by the expression

$$k_{exp} = k_0 + k_q[Q] \quad (3)$$

k_0 is the natural-decay rate constant. The quencher concentration is [Q].

The maximum concentrations of quenchers used (as the *N*-acetyl derivatives) in the low-viscosity experiments are as follows: tyrosine, 6.2×10^{-5} M; tryptophan, 4.1×10^{-5} M; histidine, 2.9×10^{-5} M. The maximum concentrations of quenchers used (as the *N*-acetyl derivatives) in the high-viscosity experiments are as follows: tyrosine, 1.4×10^{-4} M; tryptophan, 1.9×10^{-4} M; histidine, 2.9×10^{-4} M; methionine, 3×10^{-4} M. The concentration of 10-(carboxyethyl)flavin was 4.6×10^{-5} M.

Results and Discussion

The transient decay curves obtained in quenching experiments of I triplet quenched by increasing concentrations of *N*-acetyl derivatives of tyrosine, tryptophan, histidine, and methionine give strictly linear semilogarithmic decay plots indicating the usual first-order decay law. Such linear semilogarithmic decay plots are obtained in all experiments, at both low and high viscosities, with these three amino acids as well as with *N*-acetylmethionine. In all experiments extrapolation of the flavin triplet optical densities to time zero in the presence and absence of quenchers yields the same [Q] = 0 initial triplet concentration. This general outcome indicates the absence of static, immediate (and, therefore, diffusion-independent) quenching processes. The second-order quenching constants k_q are obtained from the linear plots of k_{exp} vs. [Q]. The k_q values for the three CIDNP-active free amino acids (N-acetylated) are summarized in Tables I, II, and III. At the lowest viscosity (in aqueous solution) the k_q values for quenching by tyrosine and *tryptophan* are quite similar (e.g., for Tyr at 293 K and pH 7, $k_q = 9.0 \times 10^8$ M^{-1} s^{-1}; for Trp, $k_q = 1.09 \times 10^9$ M^{-1} s^{-1}). The same outcome is obtained at higher viscosities (e.g., in 67% sucrose solution). These important results indicate that ^3D does not discriminate between the side chains of Tyr and Trp and that the intensity of the Tyr or Trp polarization in proteins can, in principle, allow one to compare their extents of exposure to the accessibility probe. The quenching rate constants for free histidine (Table III) in neutral or basic solutions are lower than the rate constants for Tyr or Trp. In the case of water solutions at pH 7, this factor amounts to a 7-fold difference. This difference increases at higher viscosities. These results indicate that comparison of histidine and tyrosine or tryptophan accessibilities needs some caution and requires operation under conditions of excess ^3D (see below).

The quenching by the tyrosine peptides (Table IV) shows a small dependence on the number of tyrosine side chains, with

Table I: Second-Order Quenching Constants of 10-(Carboxyethyl)flavin Triplet by *N*-Acetyltyrosine[a]

	temp (K)	k_q (M^{-1} s^{-1})		temp (K)	k_q (M^{-1} s^{-1})
H_2O, pH 2.9	293	7.5×10^8 (1.0)	55% sucrose	295	1.8×10^8 (25)
	273	4.5×10^8 (1.8)	67% sucrose	295	8.2×10^7 (193)
H_2O, pH 7.0	293	9.0×10^8		273	7.1×10^7 (1350)
	273	5.3×10^8	50% glycerol	233	2.9×10^7
H_2O, pH 9.2	293	8.7×10^8	70% ethylene glycol	296	2.1×10^8 (3.9)
	273	3.7×10^8		261	7.7×10^7 (40)
H_2O, pH 10.5	293	5.6×10^8		231	1.9×10^7 (600)
	273	3.6×10^8		201	1.1×10^6 (6×10^4)
D_2O	295	1.1×10^9		181	1.4×10^4 (2×10^7)
	273	4.1×10^8		171	1.2×10^3 (2×10^9)
				161	7.4×10^2 (2×10^{12})

[a] In H_2O, at pH 7.0, unless indicated otherwise. Viscosity values (in cP) are indicated in parentheses.

Table II: Second-Order Quenching Constants of 10-(Carboxyethyl)flavin Triplet by *N*-Acetyltryptophan[a]

	temp (K)	k_q (M^{-1} s^{-1})		temp (K)	k_q (M^{-1} s^{-1})
H_2O	295	1.09×10^9	67% sucrose	295	5.0×10^7
	273	6.5×10^8		273	9.4×10^6
D_2O	295	8.9×10^8	50% glycerol	233	1.6×10^7
	273	4.8×10^8	70% ethylene glycol	295	3.3×10^8
30% sucrose	295	4.9×10^8 (3.0)		261	8.7×10^7
40% sucrose	295	3.6×10^8 (5.6)		231	8.6×10^6
50% sucrose	295	2.8×10^8 (14)		201	1.1×10^6
55% sucrose	295	2.0×10^8 (25)		181	2.9×10^4
60% sucrose	295	1.6×10^8 (51)		171	2.2×10^4
				161	$<3 \times 10^3$

[a] In H_2O, at pH 7.0, unless indicated otherwise. Viscosity values (in cP) are indicated in parentheses.

Table III: Second-Order Quenching Constants of 10-(Carboxyethyl)flavin Triplet by *N*-Acetylhistidine[a]

	temp (K)	k_q (M^{-1} s^{-1})		temp (K)	k_q (M^{-1} s^{-1})
H_2O, pH 7.0	293	1.4×10^8	55% sucrose	294	2.4×10^7
	273	8.7×10^7	67% sucrose	294	$<10^5$
H_2O, pH 2.9	293	0		273	$<10^5$
	273	0	50% glycerol	233	1.6×10^6
H_2O, pH 9.2	293	1.7×10^8	70% ethylene glycol	294	1.2×10^7
	273	1.0×10^8		261	2.8×10^6
H_2O, pH 7.0, 0.15 M CsCl	293	2.2×10^8		231	3.7×10^5
D_2O, pH 7.0	294	9.2×10^7		201	8.2×10^3
	273	5.5×10^7			

[a] In H_2O, at pH 7.0, unless indicated otherwise.

Table IV: Second-Order Quenching Constants of 10-(Carboxyethyl)flavin Triplet by Peptides and by BPTI at pH 7

substrate	temp (K)	k_q (10^9 M^{-1} s^{-1})	substrate	temp (K)	k_q (10^9 M^{-1} s^{-1})
Tyr-Gly	295	1.1	BPTI	293	0.72
	273	0.50		273	0.41
Tyr-Glu	295	0.54	glutathione (S–S, oxidized)	294	0.029
	273	0.30	glutathione, S-carboxymethylated	294	0.014
Tyr-Tyr	295	1.0	His-Gly	291	0.090
Tyr-Tyr-Tyr	293	1.30	His-Ala	291	0.060
hexatyrosine	293	1.40			
	273	0.53			

Table V: Second-Order Quenching Constants of 10-(Carboxyethyl)flavin Triplet by *N*-Acetylmethionine

	temp (K)	k_q (M^{-1} s^{-1})		temp (K)	k_q (M^{-1} s^{-1})
H_2O	294	3.3×10^7	70% ethylene glycol	295	1.1×10^7
	273	1.0×10^7		263	3.7×10^6
D_2O	295	2.3×10^7		233	1.1×10^6
	273	1.2×10^7		203	5.4×10^4
67% sucrose	295	4.6×10^6		183	~0

k_q increasing slowly in the order Tyr, Tyr-Tyr, Tyr-Tyr-Tyr, and hexatyrosine. This increase is however very moderate, supporting the conclusion that the k_q values of Tables I, II, and IV for Tyr (and Trp) side chains are probably close to the limiting diffusion-controlled values. Bovine pancreatic trypsin inhibitor (BPTI) is the only protein included in this study. Previous investigations [see, e.g., Muszkat et al. (1982, 1983)] indicate the presence of one completely exposed Tyr side chain (Tyr-10) and one less exposed residue (Tyr-21). Considering the increase in molecular size in going from free tyrosine to BPTI, the value of $k_q = 0.72 \times 10^9$ M^{-1} s^{-1} observed at 293 K suggests also a diffusion-controlled situation. Histidine side chains (Tables III and IV) show some sensitivity to their chemical surrounding (cf. k_q values for His, His-Gly, and His-Ala). This situation is probably due to an isoenergetic H-transfer process (see below).

The k_q values obtained for the sulfur-containing side chains *N*-acetylmethionine (Table V), *S*-(carboxymethyl)cysteine,

Table VI: Quenching of Triplets of Flavins by *N*-Acetylhistidine at pH 7 and 293 K[a,b]

flavins	τ_0 (μs)	k_q (M^{-1} s^{-1})
I	54.4	1.4×10^8
II	63	4.0×10^8
III	49	1.5×10^8
III Et ester	68.2	3.8×10^8

[a] τ_0, natural first-order lifetime. [b] I, 6,7-dimethyl-10-(carboxyethyl)isoalloxazine; II, 6,10-dimethylisoalloxazine; III, 3-(carboxymethyl)-6,10-dimethylisoalloxazine.

and the cystine of glutathione (Table IV) have considerable practical significance in the interpretation of photo-CIDNP results of proteins. Quenching by *N*-acetylmethionine is slower by factors of 30–70 than that by Tyr or Trp and some 5-fold slower than quenching by *N*-acetylhistidine. Thus, we can conclude that in the case of Tyr or Trp polarization the presence of sulfur-containing surface side chains should not exert any significant effect on the observed polarization. On the other hand, histidine polarization could probably be weakened by the presence of neighboring sulfur-containing side chains. Again, the presence of excess ^3D could prevent any bias in comparisons of Tyr, Trp, and His side chain accessibilities.

Several factors influence the quenching reactivity of histidine side chains. In addition to the moderate effects of the chemical environment mentioned above, histidine reactivity is strongly dependent on protonation of the imidazole ring. At pH 2.9 (Table III) no reactivity can be demonstrated, while maximum reactivity is observed in slightly basic solutions (pH 9.2). The reactivity in neutral solution is somewhat lower. The results of Table VI seem to indicate some retardation by the negative carboxylate groups of flavins I and III, when compared to II or to the ethyl ester of III. In line with this conclusion is the moderate acceleration observed for quenching of I triplet in the presence of electrolytes (e.g., 0.15 M CsCl, Table III). These results point to effects of electrostatic factors. The relative inefficiency of flavin triplet quenching by histidine side chains and the sensitivity of this process to several external factors suggest that this process is less energetically favored than the corresponding quenching by tyrosine or tryptophan side chains. The energy change E_Q in a process such as (1) equals the triplet energy of ^3D, E_T, less the bond energy difference B of AH and DH·.

$$E_Q = E_T - (B_{AH} - B_{DH\cdot}) \quad (4)$$

As far as eq 4 is concerned, the three side chains differ only in the value of B_{AH}, which in histidine is very probably larger than in Tyr and Trp, while the other two factors, E_T and B_{DH}, are necessarily constant. Only in the case of isoenergetic quenching ($E_Q \sim 0$) does the rate decrease below the diffusion-controlled limit [for a related H transfer in an energy-limited system, see Muszkat & Weinstein (1975, 1976a,b)]. As expected for fast bimolecular processes, the rates of quenching of flavin triplets by side chains of amino acids are strongly viscosity dependent over the whole range of viscosities studied, e.g., over the $1-10^3$ cP range (sucrose solutions) or over the $1-10^{12}$ cP range (covered by cooling 70% ethylene glycol solutions). Comparing the k_q values for water solutions at 295 and 273 K (Tables I–III and V) shows that pure temperature-dependent effects are small. These effects are to be ascribed to low activation energies on the order of 4 kcal/mol (e.g., *N*-acetyltyrosine, 4.2 kcal/mol; *N*-acetyltryptophan, 3.6 kcal/mol; *N*-acetylhistidine, 3.5 kcal/mol). Over the same temperature range a viscosity increase to 1.3×10^3 cP, in 67% sucrose at 273 K, results in very much larger effects (see, e.g., Tables I and II). The gross features of the viscosity dependence seem to be similar in all the four *N*-acetyl amino acids studied (Figure 1) despite the difference in the rate constant values between Tyr and Trp on one hand and His and Met on the other. All these results show very clearly that flavin triplet quenching is a purely diffusion-dependent process. No significant contributions from diffusion-independent processes such as static quenching or from long-range quenching (due to a tunneling-controlled situation) can be detected. Indeed, the small deuterium kinetic isotope effects reported in Tables I–III show that, under usual conditions, the contribution of the tunneling path to the hydrogen atom transfer reaction is relatively unimportant.

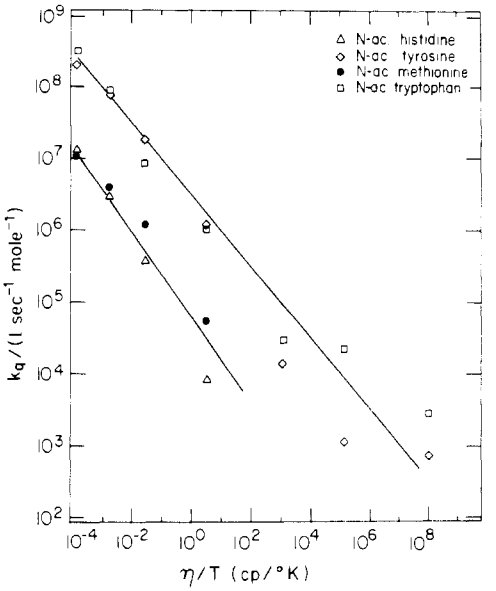


FIGURE 1: Logarithmic plot of the dependence of 10-(carboxyethyl)flavin triplet quenching rate constants (k_q) on η/T (temperature-reduced viscosity in cP/K) for the *N*-acetyl derivatives of tyrosine, tryptophan, histidine, and methionine. The solvent is 70% aqueous ethylene glycol.

Conclusions

This work provides evidence for the validity of the use of photo-CIDNP measurements for the qualitative evaluation and comparison of exposure of each of the CIDNP-active side chains (Tyr, Trp, and His). We thus see that, for a protein having both Tyr and Trp side chains in proximity, the assessment and comparison of side chain exposure under all triplet dye concentration regimes can be based on intensities of observed CIDNP signals. Histidine side chains are somewhat less reactive as flavin triplet quenchers. Thus, conclusions about the exposure of this side chain are valid only in the presence of an excess of flavin triplets [cf. Muszkat et al. (1984a)], in order to prevent competition by any neighboring Tyr, Trp, or Met side chains. The recent observation of simultaneous polarization in Tyr, Trp, and His side chains in the *Naja naja atra* cobrotoxin [Figure 2; cf. Muszkat et al. (1984b)] is fully consistent with these conclusions. The polarized residues in this short postsynaptic neurotoxin are Trp-29, His-33, and Tyr-39. These side chains all occupy relatively close positions at the active site loop. His-33 is at the center of the active site while Trp-29 and Tyr-39 are situated at its two sides, and the deletion positions in this neurotoxin are 32 and 34–36. Evidently, this simultaneous polarization obtained from one small region of a molecule possessing four disulfide bridges is thus a direct experimental proof for the consistency of the above conclusions, which have

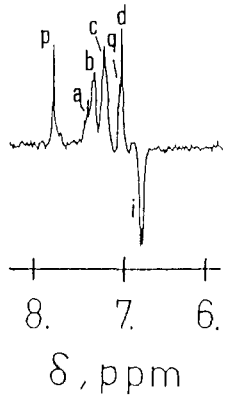


FIGURE 2: Aromatic proton region of 270-MHz photo-CIDNP spectrum of *Naja naja atra* cobrotoxin, 1.5 mM, at pH 7.6 in the presence of 0.4 mM 10-(carboxyethyl)flavin: p and q, C-2 and C-4 protons of His-33; i, C-3 and C-5 protons of Tyr-39; a, b, c, and d, C-7, C-4, C-6, and C-2 protons, respectively, of Trp-29 [taken from Muszkat et al. (1984b)].

been derived from the present triplet quenching study.

Registry No. I, 20781-13-9; II, 5618-84-8; III, 86533-03-1; IV, 72184-36-2; Tyr-Gly, 673-08-5; Tyr-Glu, 2545-89-3; Tyr-Tyr, 1050-28-8; Tyr-Tyr-Tyr, 7390-78-5; hexa-Tyr, 6934-38-9; His-Gly, 2578-58-7; His-Ala, 16874-75-2; *N*-acetyltyrosine, 537-55-3; *N*-acetyltryptophan, 1218-34-4; *N*-acetylhistidine, 2497-02-1; trasylol, 9087-70-1; glutathione (oxidized), 27025-41-8; glutathione (S-carboxymethylated), 10463-61-3; *N*-acetylmethionine, 65-82-7.

REFERENCES

Ahmad, I., Cusanovich, M. A., & Tollin, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6724–6728.

Barber, E. J. (1966) *J. Natl. Cancer Inst. (U.S.) 21*, 219–239.

Bell, R. P. (1980) *The Tunnel Effect in Chemistry*, Chapman and Hall, London.

Birks, J. B. (1970) *Photophysics of Aromatic Molecules*, Wiley-Interscience, London.

Bruhn, H., Nigam, S., & Holzwarth, J. F. (1982) *Faraday Discuss. Chem. Soc. 74*, 129–140.

Draper, R. D., & Ingraham, L. L. (1970) *Arch. Biochem. Biophys. 139*, 265–268.

Eastman, J. W., & Rosa, E. J. (1968) *Photochem. Photobiol. 7*, 189–201.

Föry, W., MacKenzie, R. E., & McCormick, D. B. (1968) *J. Heterocycl. Chem.* 5, 625–640.

Heelis, P. F. (1982) *Chem. Soc. Rev. 11*, 15–39.

Heelis, P. F., & Phillips, G. O. (1979) *Photobiochem. Photobiophys. 1*, 63–70.

Heelis, P. F., Parsons, B. J., Phillips, G. O., & McKellar, J. F. (1979) *Photochem. Photobiol. 30*, 343–347.

Holzwarth, J. F. (1982) *Faraday Discuss. Chem. Soc. 74*, 393.

Jacobson, K. A., Muszkat, K. A., & Khait, I. (1983) *J. Chem. Soc., Chem. Commun.*, 1384–1386.

Lerman, C. L., & Cohn, M. (1980) *Biochem. Biophys. Res. Commun. 97*, 121–125.

McCord, E. F., Bucks, R. R., & Boxer, S. G. (1981) *Biochemistry 20*, 2880–2888.

McCormick, D. B. (1977) *Photochem. Photobiol. 26*, 169–182.

McCormick, D. B., Falk, M. C., Rizzuto, F., & Tollin, G. (1975) *Photochem. Photobiol. 22*, 175–181.

Moore, W. M., McDaniels, J. C., & Hen, J. A. (1977) *Photochem. Photobiol. 25*, 505–512.

Müller, F., van Schagen, C. G., & Kaptein, R. (1980) *Methods Enzymol. 66*, 385–416.

Muszkat, K. A. (1977a) *Chem. Phys. Lett. 49*, 583–587.

Muszkat, K. A. (1977b) *J. Chem. Soc., Chem. Commun.*, 872–873.

Muskzat, K. A., & Weinstein, M. (1975) *J. Chem. Soc., Chem. Commun.*, 143–144.

Muszkat, K. A., & Weinstein, M. (1976a) *Z. Phys. Chem. (Munich) 101*, 105–114.

Muszkat, K. A., & Weinstein, M. (1976b) *J. Chem. Soc., Perkin Trans. 2*, 1072–1074.

Muszkat, K. A., & Gilon, C. (1977) *Biochem. Biophys. Res. Commun. 79*, 1059–1064.

Muszkat, K. A., & Gilon, C. (1978) *Nature (London) 271*, 685–686.

Muszkat, K. A., & Khait, I. (1979) *Bull. Soc. Chim. Belg. 88*, 933–939.

Muszkat, K. A., Weinstein, M., & Gilon, C. (1978) *Biochem. J. 173*, 993–996.

Muszkat, K. A., Weinstein, S., Khait, I., & Vered, M. (1982) *Biochemistry 21*, 3775–3779.

Muszkat, K. A., Weinstein, S., Khait, I., & Vered, M. (1983) *Biopolymers 22*, 387–390.

Muszkat, K. A., Khait, I., & Weinstein, S. (1984a) *Biochemistry 23*, 5–10.

Muszkat, K. A., Khait, I., Hayashi, K., & Tamiya, N. (1984b) *Biochemistry 23*, 4913–4920.

Muus, L. T., Atkins, P. W., McLauchlan, K. A., & Pedersen, J. B., Eds. (1977) *Chemically Induced Magnetic Polarization*, Reidel, Dordrecht, The Netherlands.

Tegner, L., & Holmstrom, B. (1966) *Photochem. Photobiol. 5*, 223–226.

Vaish, S. P., & Tollin, G. (1970) *Bioenergetics 1*, 181–192.