EFFECT OF COMPLEXATION OF FLAVIN RADICAL WITH TRYPTOPHAN ON ELECTRON TRANSFER RATES: A MODEL FOR FLAVIN-PROTEIN INTERACTIONS†

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SUMMARY

The rates of oxidation of lumiflavin radical by ferricyanide, indole radical and oxygen are decreased by factors of four to ten as a result of complexation with tryptophan. Tyrosine, methionine and glycine were found not to measurably alter the flavin radical reactivity. Similar results were obtained using flavinyl peptides in which tryptophan or methionine were covalently linked to the flavin. These observations suggest that one of the consequences of the interaction between the flavin and a tryptophan side chain in the coenzyme binding site of the flavodoxins is to deactivate the semiquinone form of the enzyme towards oxidizing agents, thereby increasing its stability.

Recent x-ray structure studies of flavodoxins from Desulfovibrio vulgaris (1,2) and Clostridium MP (3,4) have shown that the FMN cofactor is highly buried within the protein and interacts with tyrosine and tryptophan side chains in the former enzyme and methionine and tryptophan side chains in the latter. Experiments utilizing chemical modification (5) and protein fluorescence (6) have implicated a tryptophan side chain in the flavin binding site of the Azotobacter flavodoxin as well. As part of a study of the pathway of electron transfer in the flavodoxins, we have investigated the effect of complexation of unbound flavin with these amino acids on the rates of several flavin radical reactions. The results of these experiments demonstrate that the proximity of the indole side chain of tryptophan to the isoalloxazine ring of the flavin can act to significantly decrease the rates of electron transfer reactions of the flavin radical. Tyrosine and methionine side chains had no affect on these rates. These observations are consistent with the fact that the rates of

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oxidation of the semiquinone forms of the flavodoxins are appreciably lower than the corresponding rates of the unbound coenzyme (7), and suggest that the indole side chain of tryptophan plays a role in this phenomenon.

MATERIALS AND METHODS

The rates of oxidation of the radical form of lumiflavin by oxygen and ferricyanide were determined utilizing flash photolysis as previously described (8). The source of electrons for reduction of the triplet state of the flavin was either an amino acid (tyrosine, tryptophan or methionine) or EDTA. Experiments were carried out at room temperature in 0.1 M phosphate buffer, pH 7.0 (except as noted otherwise). At this pH, the radical is predominantly in the neutral form, although O_2 oxidation proceeds via the small amount of anion radical which is present in equilibrium (8). In one set of experiments utilizing tryptophan as the reductant, oxidizing agent was excluded. In this case the radical decay was predominantly due to the reverse electron transfer reaction between oxidized tryptophan and reduced lumiflavin. Radical disproportionation appeared to be minimal.

RESULTS

Draper and Ingraham (9) have shown that the amino acids tyrosine and tryptophan form reasonably strong complexes with the semiquinone forms of FMN and riboflavin (formation constants $\sim 10^2 M^{-1}$). Although methionine was not investigated, cystine and oxidized glutathione were found to participate in complex formation. Cystine in particular seems to form a rather strong complex. Thus, if such complexation affects the rates of reaction of the radical, it should be possible to observe this by studying the decay kinetics of the semiquinone in the presence of increasing amounts of amino acid. Figure 1 shows the results of an experiment in which oxygen was the oxidant and tryptophan the reductant, and the concentration of the amino acid varied over approximately three orders of magnitude. It is seen that at the lowest tryptophan concentration, reasonably good pseudo-first order kinetics were obtained. The slope of this line is in excellent agreement with earlier results using 2,6-

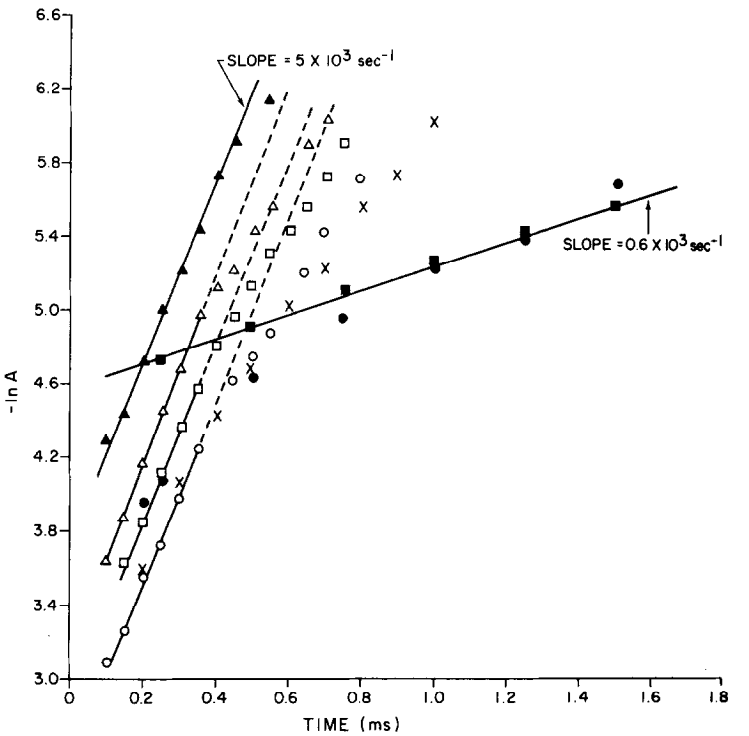

Figure 1. Pseudo-first order rates of O_2 oxidation of lumiflavin semiquinone in the presence of varying amounts of tryptophan. Measurements made at 560 nm in air-saturated 0.1 M phosphate buffer, pH 7.0; lumiflavin concentration = 2×10^{-4}M. $k_{apparent} = 5 \times 10^3 sec^{-1}$ at 1×10^{-5}M trp; $k_{apparent} = 0.6 \times 10^3 sec^{-1}$ at 5×10^{-2}M trp.

▲ 1×10^{-5}M trp
△ 6.3×10^{-5}M trp
□ 1×10^{-4}M trp
○ 1×10^{-3}M trp
X 8.3×10^{-3}M trp
● 2.5×10^{-2}M trp
■ 5×10^{-2}M trp

dimethylphenol as the reductant (8). As the tryptophan concentration was increased, strong deviations from first order kinetics were observed. At the highest tryptophan concentration (5×10^{-2}M), first order behavior was again obtained, although there was an approximately ten-fold decrease in oxidation rate. Note that the decay curve generated in the presence of 2.5×10^{-2}M tryptophan is biphasic, one component decaying at a rate approximately that obtained at lower tryptophan concentrations and the other at a rate approaching that obtained at the highest tryptophan concentration. These results are consistent with a mechanism involving two forms of the lumiflavin radical which react with oxygen at rates which differ by about a factor of ten. Utilizing

the formation constants determined by Draper and Ingraham (9) for tryptophan complexation of the riboflavin semiquinone, it is possible to calculate that approximately 85% of the radical should be complexed in the presence of 5×10^{-2}M tryptophan. Thus, it seems reasonable to identify the two forms of the radical indicated by the results of Fig. 1 with the free and complexed lumiflavin species.

Tyrosine (up to a concentration of 10^{-2}M), methionine (up to a concentration of 10^{-1}M) and glycine (up to a concentration of 10^{-1}M) were found to be without any appreciable affect on the rate of lumiflavin radical oxidation by oxygen. Furthermore, addition of 10^{-2}M tyrosine or 10^{-1}M methionine to a lumiflavin solution containing 5×10^{-2}M tryptophan caused no further decrease in reaction rate. The results with tyrosine are consistent with a previously reported observation (8) utilizing 2,6-dimethylphenol as reductant. It is significant that Draper and Ingraham (9) have determined that the formation constant of the complex between tyrosine and riboflavin radical is approximately equal to that of tryptophan. Thus, it would appear reasonable to conclude that complexation of lumiflavin radical with tyrosine is occurring but that this has no affect on the rate of oxidation.* The results with methionine indicate that, if a complex is in fact formed, this interaction also does not alter the reactivity of the lumiflavin radical towards oxygen. All of the data support the conclusion that the rate decrease is a consequence of interactions between the indole side chain of tryptophan and the isoalloxazine ring of the flavin semiquinone (10).

Ferricyanide oxidation of lumiflavin radical was found to be similarly retarded by increasing concentrations of tryptophan (Fig. 2). In this case, the rate decrease was approximately sevenfold. The results at low amino acid concentrations again were in good agreement with previous work (8). As was the

*Unfortunately, the limited solubility of tyrosine precluded using concentrations higher than 10^{-2}M. Thus, if the formation constant of the lumiflavin radical-tyrosine complex were in fact somewhat smaller than that of tryptophan, it is possible that rate effects would not be observed in the present experiments. We have, however, determined that 3,4-dihydroxyphenylalanine at a concentration of 5×10^{-2}M has no effect on the O_2 oxidation rate.

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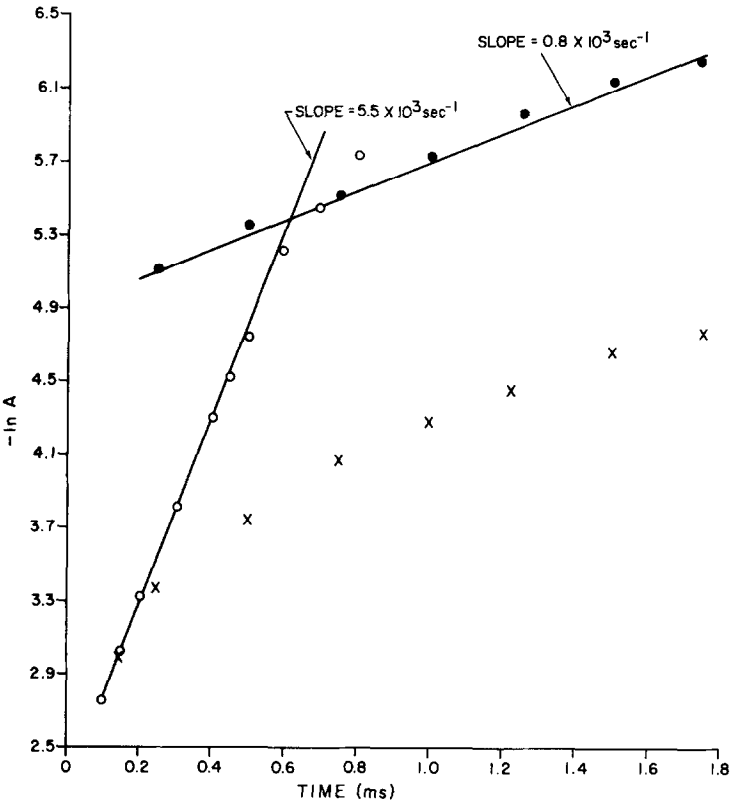

Figure 2. Pseudo-first order rates of ferricyanide (1×10^{-5}M) oxidation of lumiflavin semiquinone in the presence of varying amounts of tryptophan. Experimental conditions same as in Figure 1 except that samples were deoxygenated $k = 5.5 \times 10^{8}M^{-1}sec^{-1}$ at 2.5×10^{-3}M trp; $k = 0.8 \times 10^{8}M^{-1}sec^{-1}$ at 5×10^{-2}M trp.

○ 2.5×10^{-3}M trp
× 1×10^{-2}M trp
● 5×10^{-2}M trp

case with O_2 oxidation, tyrosine (10^{-2}M) and methionine (10^{-1}M) were without affect on the radical decay rate. We have also determined that 5-hydroxytryptophan decreases the rate of ferricyanide oxidation of the lumiflavin semiquinone in a manner similar to tryptophan. In this case, the limiting rate constant (at 1.5×10^{-1}M) was found to be $0.6 \times 10^{8}M^{-1}sec^{-1}$ (compared to $0.8 \times 10^{8}M^{-1}sec^{-1}$ for tryptophan).

Figure 3 shows that the complexation of lumiflavin radical with tryptophan acts to significantly decrease (approximately fourfold) the rate of the reverse electron transfer reaction from the oxidized amino acid.

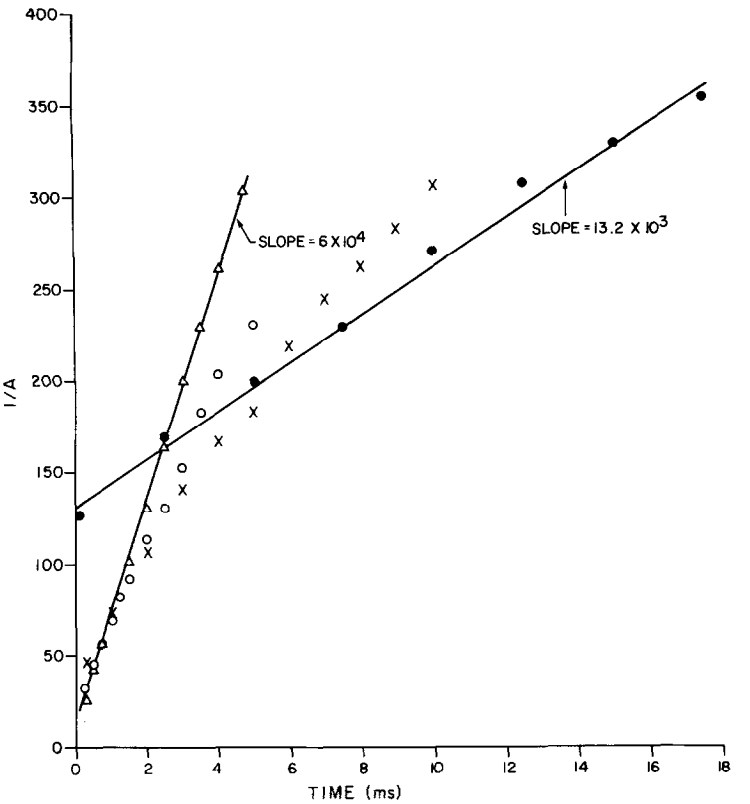

Figure 3. Second order rates of lumiflavin semiquinone decay in the absence of added oxidizing agent and in the presence of varying amounts of tryptophan. Experimental conditions same as in Figure 1 except that samples were deoxygenated. $k = 2.5 \times 10^{8} M^{-1} sec^{-1}$ at 8×10^{-4}M trp; $k = 6.2 \times 10^{7} M^{-1} sec^{-1}$ at 5×10^{-2}M trp.

Δ 8×10^{-4}M trp
O 1.3×10^{-2}M trp
X 2.5×10^{-2}M trp
● 5×10^{-2}M trp

We have also carried out several experiments using flavinyl peptides (i.e. 7,8-dimethyl-10 (ω-carboxyalkyl) isoalloxazine peptides) (11) in which the amino acid residues covalently joined by peptide linkage to the carboxyl at the end of the 10-alkyl side chain were tryptophan or methionine.† Föry et al. (12) have shown that the amino acid group tends to fold back onto the flavin ring in compounds of this type. Furthermore, at least in the case of the tyrosyl and tryptophyl peptides, the flavin fluorescence is highly quenched (13).

Flash photolysis of the methionyl peptide (with five methylene groups in the 10-position), in 0.1 M formate buffer, pH 4.5, yielded appreciable amounts

of semiquinone only in the presence of high concentrations of EDTA (~10 mM). No semiquinone was obtained in the absence of EDTA. This could be due either to ground state quenching or to an extremely rapid reverse electron transfer rate. Although quantitative comparisons were not made, it was quite clear that radical yields were appreciably lower with the flavin methionyl peptide than with ordinary flavins. The rate constant for the disproportionation of the neutral semiquinone was found to be $5.0 \times 10^{9} M^{-1} sec^{-1}$. This can be compared to values of $6.2 \times 10^{9} M^{-1} sec^{-1}$ for the neutral lumiflavin radical (8) and $3.5 \times 10^{9} M^{-1} sec^{-1}$ for the neutral riboflavin radical (G. Tollin, unpublished data), obtained under similar conditions. For ferricyanide oxidation of the neutral radical, the rate constant is $5.0 \times 10^{8} M^{-1} sec^{-1}$ (the value for lumiflavin radical is $5.5 \times 10^{8} M^{-1} sec^{-1}$) and for O_2 oxidation, measured at pH 7.0 in air-saturated 0.1 M phosphate buffer, the pseudo-first order rate constant is $4.3 \times 10^{3} sec^{-1}$ (the value for lumiflavin radical* is $5 \times 10^{3} sec^{-1}$). Thus, it is seen that even when the methionine side chain is constrained by covalent attachment to be in the vicinity of the flavin ring, no appreciable affect on radical reactivity is observed.

Flash photolysis of the tryptophyl peptide (with five methylene groups in the 10-position), in 0.1 M formate buffer, pH 4.5, in the presence of 10 mM EDTA gave a radical signal which was approximately one-fourth as large as that obtained with the corresponding methionyl peptide.† The rate constant for disproportionation of the neutral semiquinone is approximately $2 \times 10^{9} M^{-1} sec^{-1}$. For O_2 oxidation (measured in air-saturated 0.1 M phosphate buffer, pH 7.0) the pseudo-first order rate constant is $1.3 \times 10^{3} sec^{-1}$. Thus, both of these reaction rates are significantly diminished in the tryptophyl peptide. On the other hand, the rate constant for ferricyanide oxidation of the neutral radical was virtually unchanged ($k = 5 \times 10^{8} M^{-1} sec^{-1}$). This rather surprising result may be a consequence of differences in the geometry of the flavin-tryptophan interaction

†The radical yields obtained with a tryptophyl peptide having two methylene groups in the 10-position were exceedingly small. This correlates with the higher degree of fluorescence quenching observed with this compound (13).

in the peptide as compared to the free components. That ferricyanide and O_2 oxidations of flavin radicals respond differently to modifications of the flavin ring system has been shown previously (7,8). For example, protonation at the N-5 position greatly diminishes the O_2 rate (>10^4 fold) whereas changes at the 7 and 8-positions decrease the ferricyanide rate (2-3 fold) without affecting the O_2 rate. It is perhaps significant that the structure of the folded conformation of the flavinyl tryptophan deduced by Föry et al. (12) from nmr studies has appreciable overlap between the two ring systems at the N5-position and little or no overlap at the 7 and 8-methyl groups.

CONCLUSIONS

The present experiments have shown that the proximity of the indole ring of tryptophan specifically decreases the rates of several reactions of the lumiflavin radical. Whether steric or electronic effects (or both) are involved in this rate decrease cannot be unambiguously decided at present. The results are consistent with the observation that the reactivity of the semiquinone forms of the flavodoxins is much lower than that of the free coenzyme (7,14). However, in the case of the flavoproteins, the rate decreases are considerably larger than those observed here (e.g. the rate of ferricyanide oxidation is approximately 10^4 times less with the flavodoxins than with free FMN). This is perhaps not surprising, in view of the facts that other interactions between the flavin ring and the protein are present in the enzymes and that the geometry of the indole-isoalloxazine interaction is most likely different in the proteins than it is in the free flavin-tryptophan complex or the flavinyl peptide. It is certainly reasonable to conclude, however, that at least one of the consequences of the indole-flavin interaction in the flavodoxins is a lowering of the reactivity of the semiquinone species towards oxidizing agents.*

*This comparison assumes no difference in the semiquinone pK values.

*Wu et al. (15) have shown that the close proximity of an aromatic ring (e.g. tyrosine or tryptophan) acts to make the flavin reduction potential more positive by 0.05-0.06 V. However, the flavodoxin potentials are, in general, more negative than that of free FMN (16). It is also of interest that complexation of oxidized flavin with tyrosine and tryptophan decreases the rate of its reduction by NADH (17).

Inasmuch as these enzymes function by shuttling between the semiquinone and fully-reduced forms, such a stabilization would be a significant factor in catalysis. It is important to note that tryptophan is the conserved amino acid residue in the coenzyme binding sites of the two flavodoxins whose structures have been determined, and is probably in the binding site of the Azotobacter flavodoxin as well (1-6). The possible role of the binding site tyrosine and methionine side chains in these enzymes awaits elucidation. Perhaps these amino acids influence the reactivity of the fully-reduced form of flavin. Experiments to test this are presently underway.

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