Tetrahedron Letters No. 24, pp 2073 - 2076, 1977. Pergamon Press. Printed in Great Britain.

INTERACTION OF ALIPHATIC AMINO ACIDS WITH RIBOFLAVIN

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(Received in USA 27 September 1976; received in UK for publication 3 May 1977)

Flavins are known to form association complexes with a large variety of substances of biological relevance. Charge transfer character has been postulated for most of these complexes, with the flavins acting as acceptors.[1] Complexes of riboflavin (Rf) with amino acids have been reported several times[1,2]. However, with the exception of tryptophan[3] no association constants have been determined for such complexes. Nevertheless, the mechanism of these constants is the best way to understand the mechanism of complex interactions.

We now wish to report difference spectra and fluorescence quenching results, which allowed the determination for the first time of apparent association constants for several aliphatic amino acids and Rf. The studies were performed in aqueous solutions of Rf and amino acids. These solutions were prepared in deionized and tridistilled water. The same results were obtained when the studies were carried out in phospahte buffered solutions (pH[6]). Samples were handled in the dark in order to prevent Rf photoreactions. Absorption spectra were taken in a Cary 17 spectrophotometer and the fluorescence studies were carried out in an Aminco SPF 125 spectrophotofluorometer. Rf concentracions used were in the range 10^{-4} to 2.0×10^{-5} M. The amino acid concentrations were the highest possible in order to detect the interaction; and ranged from 0.01 to 1.0 M depending on their solubility.

The amino acids used were: glycine, alanine, valine, leucine, isoleucine, norleucine, serine and threonine; in their presence the absorption spectrum of Rf showed small changes. The principal features observed were a decrease of the absorvance at 445 nm and 265 nm and a noticeable shift to shorter wave lengths of the UV bands. No new bands could be detected for the mixtures. These changes

were more evident in the difference spectra, as it is shown in Figure 1, for a typical case.

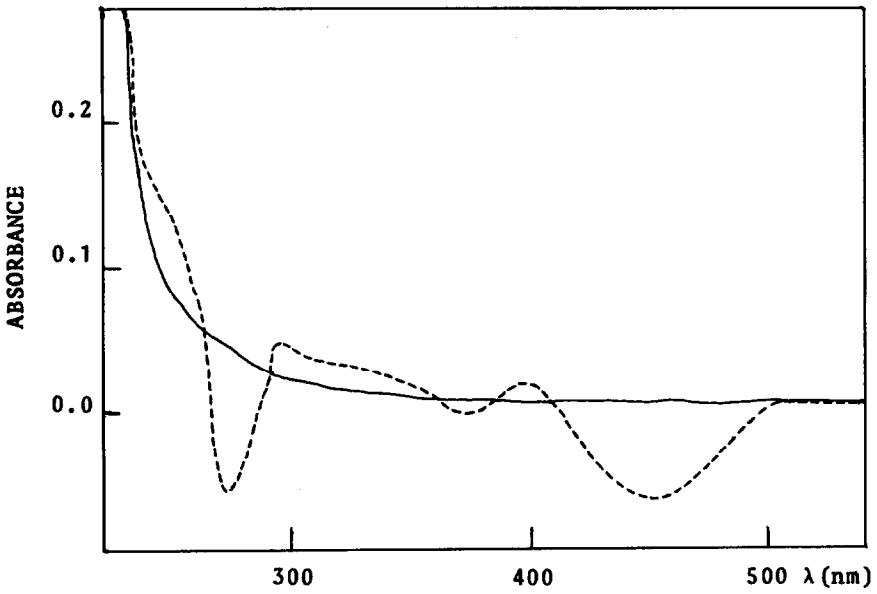

Fig. 1. Difference spectrum Rf ($7x10^{-5}$ M) + Norleucine ($7.6x10^{-2}$ M) vs. Rf ($7x10^{-5}$ M) (----); Norleucine ($7.6x10^{-2}$ M) vs. buffer (——).

Difference spectra for Rf and glycine, alanine and leucine have also been reported by Slifkin.[2] He used amino acids concentrations of 10^{-2} M. With these concentrations we were not able to reproduce those results. In all cases we needed concentrations ranging from 50 to 100 fold greater than those reported by Slifkin, to obtain measurable bands.

We tried to evaluate association constants (K_{ass}) from the difference spectra using a Rose-Drago type equation.[4] In several cases departures form linearity were observed and negative values of K_{ass} were obtained. Even in those cases in which positive values of K_{ass} were calculated it is probable that they were not accurate; this is not unexpected since the method is known to be unsatisfactory when dealing with weak complexes such as these.[5] Nevertheless, from the negative band at 445 nm we colud establish a qualitative order of increasing interaction, i.e.:

Glycine < Alanine < Valine < Leucine

Another commonly used procedure to obtain apparent association constants is the fluorescence quenching. Rf presents a strong fluorescence emission at 515 nm (uncorrected) which can be quenched by several substances.[6] In the assumption that the quenching does not proceed through and interaction with the excited state, apparent association constants for complexing in the ground state (assuming a 1:1 complex) can be calculated from Stern-Volmer plots[7]:

$$\frac{I_o}{I} = 1 + K_{ass} [Q]$$

Where I_o refers to fluorescence intensity in absence and presence of the quencher Q respectively. Values of K obtained by this method can be seen in Table I. The error quoted corresponds to the standard deviation obtained by least squares adjustment.

TABLE I

Amino Acid	K_{ass}(25°C) M^{-1}	Amino Acid	K_{ass}(25°C) M^{-1}
Glycine	0.064 ± 0.003	Isoleucine	0.47 ± 0.04
Alanine	0.10 ± 0.01	Norleucine	0.45 ± 0.03
Valine	0.14 ± 0.01	Serine	0.63 ± 0.008
Leucine	0.43 ± 0.05	Threonine	0.19 ± 0.02

The values in the Table show the same trend as the qualitative order of increasing interaction obtained by the difference spectra.

The temperature effect on K_{ass} was also studied. At 45°C we obtained the same values within the experimental errors, as could be expected for such small values of K_{ass}.

Whitten et al.[8] reported Stern-Volmer constants for the fluorescence quenching of Rf by several nucleophiles, including a value of 0.08 M^{-1} for $CH_3CO_2^-$, intermediate between those found by us for glycine and alanine. They ascribe the observed effect to an interaction with the excited state. However we think that these small apparent association constants could well account for the interaction

observed spectroscopically in the ground state.

Slifkin[2] proposed an interaction mechanism for amino acids with Rf in which the equilibrium could be attained from the stabilization of the semiquinone form of Rf by electron donation of the free amino groups to the isoalloxazine moiety. He concludes that, since the fraction of free amino groups in neutral aqueous solutions is relatively small, the equilibrium would not be reached instantaneously, but it should take several hours to be completed. This was in accordance with a time dependence of the observed spectral changes.[2] Nevertheless our results are independent of time and no spectral changes that could indicate an increase of interaction were observed after several days.

In the system Phenol-Acetate, Scheraga et al[7], suggest that, in addition to the formation of an intermolecular hydrogen bond, a hydrophobic bond is formed between the methyl group of the acetate and the benzene ring of the phenol.

We think that a similar interaction mechanism is operating in the system riboflavin-amino acids on the ground of the following evidence:

a) small association constants which increase with the bulkyness of the aliphatic chain of the amino acid.
b) lack of temperature dependence in the association constants within experimental errors.

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Acknowledgement: Thanks are given to the "Consejo Nacional de Investigaciones, Argentina" for financial support.