Intermolecular Complexes between *N*-Methyl-1,4-dihydronicotinamide and Flavines. The Influence of Steric and Electronic Factors on Complex Formation and the Rate of Flavine-Dependent Dihydronicotinamide Dehydrogenation†

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ABSTRACT: The reaction of *N*-methyldihydronicotinamide (NMNH) with flavine analogs saturates at high dihydronicotinamide concentrations. Complex formation between the reactants depends mainly on steric but not on electronic factors. Thus flavine analogs that differ up to 243 mV in their oxidation–reduction potential vary only between 0.09 and 0.17 *M* in K_d. When the flavine plane becomes blocked by bulky substituents, however, complex stability decreases by more than an order of magnitude. NMNH–flavine complexes show long wave optical absorption. The energy of the long wave transition decreases with increasing oxidation–reduction potential of the flavine as expected for charge transfer complexes. The first-order rate constants of flavine-dependent dihydronicotinamide dehydrogenation increase with increasing oxidation–reduction potential of the flavine but they are almost independent of K_d. The reaction is not subject to general acid-base catalysis. Thus flavine-dependent dihydronicotinamide dehydrogenation may be interpreted to proceed via a charge transfer complex between oxidized flavine and reduced nicotinamide. In the rate-limiting conversion of the charge transfer complex into products hydrogen is transferred directly, the rate being governed by the difference in oxidation–reduction potential between flavine and dihydronicotinamide. An alternative mechanism where the observed charge transfer complex is not on the reaction pathway appears to be improbable but cannot be eliminated.

Long wave absorbing complexes between reduced flavine and oxidized nicotinamide have been described in flavoproteins as well as model systems. They have been well characterized as charge transfer complexes by correlation of the energy of the long wave absorption with the lowest vacant orbital energies of several NAD$^+$ analogs (Sakurai and Hosoya, 1966). In flavoproteins it has been demonstrated that these charge transfer complexes are catalytic intermediates in some enzymes but not in others (Massey and Palmer, 1962; Massey and Ghisla, 1974).

Long wave absorbing complexes between oxidized flavine and reduced nicotinamide have not as yet been well characterized. They were first described for Old Yellow Enzyme (Haas, 1937) and NADH–peroxidase (Dolin, 1957). Recently it has been shown that such complexes are catalytic intermediates in several flavoenzymes (Massey et al., 1970). More recently these complexes were detected in model systems as well (Porter et al., 1973; Blankenhorn, 1975). Originally they were postulated to be of biradical nature (Massey et al., 1970), but this interpretation has since been dropped in favor of charge transfer complexation (Massey and Ghisla, 1974). On the other hand, Hemmerich (Hemmerich, 1970) proposed a covalent flavine–nicotinamide structure for the long wave absorbing species. Bruice (Bruice et al., 1971) postulated "face to face" complexes and Porter (Porter et al., 1973) suggested a charge transfer type complex.

In the present study the reaction of selected flavine derivatives with NMNH[1] has been investigated in order to provide evidence for the postulated charge transfer character of the long wave absorbing intermediates. Furthermore the factors governing the rate of flavine-dependent dihydronicotinamide dehydrogenation have been examined.

Materials and Methods

All flavine derivatives used in this study were either commercial products or synthesized according to published procedures: *S*-methyl-2-thiolumiflavine (Müller and Hemmerich, 1966), 10-methylisoalloxazine (Kuhn and Weygand, 1934), 10-methyl-5-deazaisoalloxazine (O'Brien et al., 1970), 10-2,2′-dimethylphenylisoalloxazine (Knappe, 1974), 8-nor-8-morpholinolumiflavine (according to Ehrenberg et al., 1967), 8α-oxo-3-methyllumiflavine (Salach et al., 1972). FMN (Hoffmann-La Roche) and FAD (Boehringer) were used without further purification. NMNH was prepared by the method of Holman and Wiegand (Holman and Wiegand, 1948). It could be obtained in crystalline form when the oily product was treated with diisopropyl ether at −15°. This product had a molar extinction coefficient of 7.6 × 10^3, which is slightly higher than that reported in the literature (Rafter and Colowick, 1954). Catalase and glucose oxidase were analytical grade enzymes from Boehringer. The purity of the flavine derivatives was checked by thin-layer chromatography (DC-Fertigplatten Kieselgel 60, Merck) using the solvent system 1-butanol–acetic acid–water (5:2:3).

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[1] Abbreviations used are: Fl_{ox}, oxidized flavine; $Fl_{red}H_2$, reduced flavine; Nic_{ox}^+, oxidized nicotinamide; $Nic_{red}H$, reduced nicotinamide, NMNH, *N*-methyldihydronicotinamide.

Table I: Absorption Maxima and Dissociation Constants of Flavine Analog–*N*-Methyldihydronicotinamide Complexes. First-Order Rate Constants of Flavine Analog Reduction and Polarographically Determined Flavine Analog Oxidation Reduction Potentials Measured in 0.01 *M* Tris-HCl Buffer (pH 8.0) Containing 0.5 *M* KCl.

	Flavine Analog	λ_{max} CT (nm)	K_d (*M*)	k_2 (sec^{-1})	Ox.–red. Potential pH 8 (mV)
I	10-Methyl-5-deaza-isoalloxazine	525	0.17	1.0	−380[a]
II	8-Nor-8-morpholinolumiflavine	<550	0.14	2.1	−273
III	Lumiflavine	575	0.10	7.0	−231
IV	FMN	575	0.13	7.0	−225
V	FAD	575	0.25	6.25	−223
VI	10-Methylisoalloxazine	575	0.17	14	−216
VII	7,8-Dimethyl-10-phenylisoalloxazine	575	0.24	6.7	−203
VIII	*S*-Methyl-2-thiolumiflavine	625	0.09	59	−127
IX	10-2,2′-Dimethylphenylisoalloxazine		>5.0		−120
X	8α-Oxo-3-methyllumiflavine	685	0.16	192	−30

[a] This value does not represent a true oxidation–reduction potential, but is the midpoint of the potentiometric reduction wave.

Half-wave potentials for the 2e$^-$ flavine reduction were obtained polarographically using the instrument "Polarographiestand E 354" from Metrohm in connection with the recorder "Polarecord E 261". A saturated Ag/AgCl electrode was used as reference against the dropping mercury electrode, the temperature was 20°; for potentiometric titrations at pH 8 the method of Böhme and Cramer was used (Böhme and Cramer, 1973). Spectrophotometric measurements were made with a Cary 14 and rapid kinetics with a Durrum Gibson spectrophotometer. NMNH- and *S*-methyl-2-thiolumiflavine solutions were freshly prepared prior to use, the other flavine derivatives could be stored in solution in the dark. In rapid kinetic measurements anaerobiosis of solutions was achieved by adding to 10-ml volumes in the stopped-flow reservoir syringe 50 μl of an aqueous saturated glucose solution, 10 μl of catalase (20 mg/ml), and 10 μl of glucose oxidase (24 mg/ml). All reactions were measured at 25°, in 0.01 *M* Tris-HCl buffer (pH 8.0) containing 0.5 *M* KCl. The rate constants k_2 and K_D given in Table I represent the average of at least three measurements. The mean error was determined as ±10%.

Results and Discussion

Kinetics and Mechanism of the Flavine Reduction by NMNH. NMNH was used as a NADH model because of its good water solubility; the high dissociation constants of the dihydronicotinamide–flavine complexes require a high concentration of dihydronicotinamide in order to obtain significant amounts of complex. The flavine derivatives used in this study were selected by two criteria: (1) flavine analogs that differ from the natural flavine cofactors FMN and FAD in their oxidation reduction potential, and (2) flavine analogs where access to the flavine plane is hindered by bulky substituents.

The anaerobic reduction of the flavine analogs by NMNH was followed with a stopped-flow spectrophotometer at 470 nm; the disappearance of the long wavelength absorbing complex was observed at 550–700 nm. Logarithmic plots of the absorbance changes gave straight lines for more than 90% of the reaction. The first-order rate constants proved to be identical when calculated from either 470 nm or long wavelength absorbance changes. Except for 10-2,2′-dimethylphenylisoalloxazine, saturation kinetics were obtained (Figure 1). It is probable that steric hindrance increases the K_d for the latter flavine analog to an extent that its value cannot be determined. Thus, reaction between flavine analogs and *N*-methyldihydronicotinamide may be represented as follows:

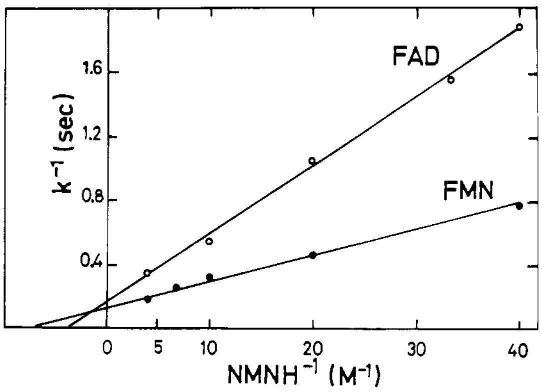


FIGURE 1: Plots of the inverse of the anaerobic rate of flavine reduction (5×10^{-5} *M*, pH 8.0) vs. the inverse of NMNH concentration.

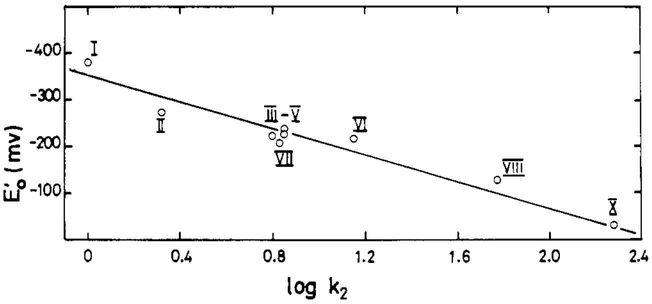


FIGURE 2: Plot of log k_2 of flavine analog reduction (5×10^{-5} *M*, pH 8.0) by NMNH vs. the flavine analog oxidation reduction potential (pH 8.0). Numbers refer to those listed in Table I.

$$\mathrm{Fl_{ox}} + \mathrm{Nic_{red}H} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Fl_{ox}} \underset{\text{complex}}{\overset{\pi}{\text{———}}} \mathrm{Nic_{red}H} \xrightarrow{k_2} \mathrm{Fl_{red}H^-} + \mathrm{Nic_{ox}^+}$$

The results are summarized in Table I. For all flavine analogs the rate of complex formation was too high to allow determination of k_1. As long as access to the flavine plane remained unhindered even a large difference in oxidation–reduction potential caused only a minor change in K_d. When access to the flavine plane became increasingly blocked, however, K_d values for the NMNH complexes increased dramatically. For 10-2,2′-dimethylphenylisoalloxazine the experiments predict a value higher than 5 *M* as compared to values of 0.25 for FAD, 0.13 for FMN, and 0.10 for lumiflavine.

The rate of product formation increased with increasing oxidation–reduction potential of the flavine analog. The plot of log k_2 vs. the flavine oxidation–reduction potential shows a linear free energy relationship (Figure 2). The rate-limiting step of the reaction has been shown to consist in the

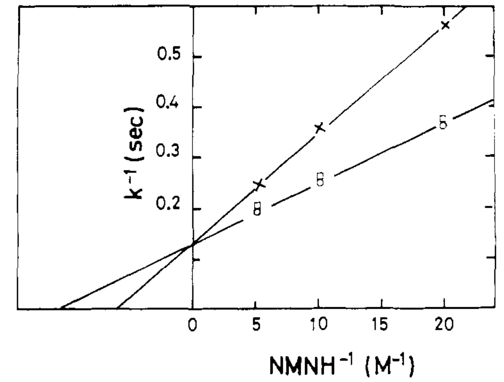


FIGURE 3: Plot of the inverse of the anaerobic rate of lumiflavine reduction (5×10^{-5} *M*, pH 8.0) vs. the inverse of NMNH concentration (○) in the presence of 0.1 *M* imidazole (□) and 0.1 *M* phenol (x).

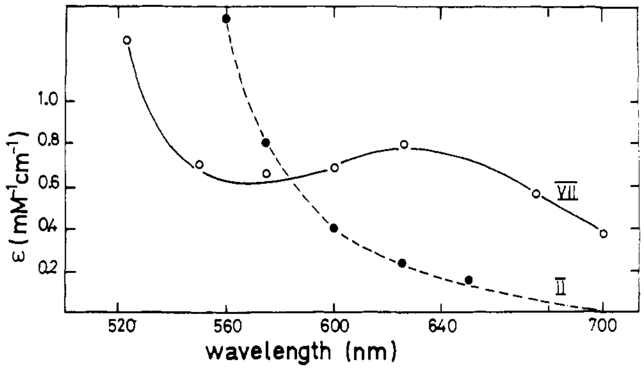


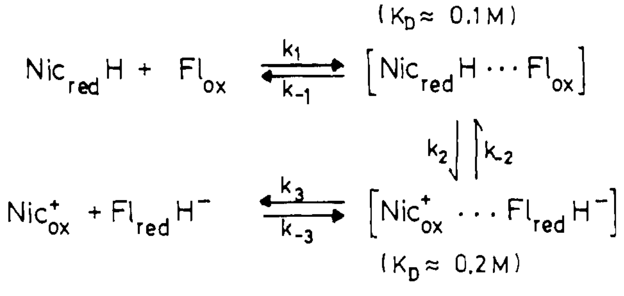
FIGURE 4: Long wavelength absorption of flavine analog-NMNH complexes. II, 8-nor-8-morpholinolumiflavine; VIII, *S*-methyl-2-thiolumiflavine.

cleavage of the C(4)-H bond in 1,4-dihydronicotinamide (Suelter and Metzler, 1960). If H^+ or H^- were not directly transferred from dihydronicotinamide to flavine the reaction should be subject to general acid base catalysis unless hydrogen atom transfer occurred. If it were transferred directly, however, the reaction should not be subject to general acid base catalysis. Imidazole (general base) or phenol (general acid) added to the reaction mixture at pH 8 in concentrations ranging from 10^{-3} to 10^{-1} *M* did affect K_d but had no effect on k_2 demonstrating absence of general acid base catalysis (Figure 3) and indicating direct hydrogen transfer. Complex formation between imidazole and flavine was insignificant whereas a K_d of 0.4 *M* for the lumiflavine-phenol complex was determined.

Direct hydrogen transfer from dihydronicotinamide to C(5) of a 5-deazaflavine has been demonstrated in a model system (Brüstlein and Bruice, 1972). Enzymatic reduction of 5-deazariboflavine from NADH by direct hydrogen transfer has also been described (Fischer and Walsh, 1974). It has to be pointed out, however, that the validity of using 5-deazaflavines as models to obtain information on the mode of hydrogen transfer from substrate toward flavine appears questionable: 5-deazaflavines are closer structural analogs to nicotinamide than to flavine (Hemmerich and Schuman-Jörns, 1973). Thus demonstration of direct hydrogen transfer is hardly surprising. The 2e$^-$ shuttle involves formation and cleavage of a kinetically stable C-H bond and stability of the semiquinone is dramatically decreased (Edmondson et al., 1972). Therefore the midpoint potential for 2e$^-$ reduction obtained by polarography at pH 8 is by far too negative (−657 mV) whereas potentiometry yields a midpoint potential of −380 mV. This latter value is similar to those found for dihydronicotinamide derivatives.

As a consequence of the rather high K_d values of the flavine-dihydronicotinamide complexes, the observed rate of flavine reduction will not only depend on the difference in oxidation-reduction potential between the reactants, but also on the K_d of the complex. The experimental results reported in this paper are in good agreement with similar data by Bruice (Bruice et al., 1971). However, these authors did not recognize the free energy relationship reported in this paper.

All flavine analogs investigated form a long wave absorbing, transient intermediate after mixing anaerobically with NMNH (Table I). Their electronic spectra very closely resemble those of the corresponding intramolecular complexes (Blankenhorn, 1975) as well as those of the complexes between NADPH and several flavoenzymes (Massey et al., 1970). For FMN and FAD the maximum of the long wave absorption is centered around 575 nm with a molar extinction coefficient of 800 M^{-1} cm^{-1}. The 8-nor-8-morpholinolumiflavine complex (Figure 4) shows no distinct long wave absorption maximum because it is masked by the absorption of the oxidized flavine chromophore (λ_{max} 478 nm). In contrast, the *S*-methyl-2-thiolumiflavine complex shows long wave absorption (λ_{max} 625 nm) clearly separated from the π-π^* transition of the oxidized flavine chromophore (λ_{max} 457 nm). Thus the observed long wave absorptions may be classified as charge transfer transitions according to Mulliken's definition (Mulliken, 1952).



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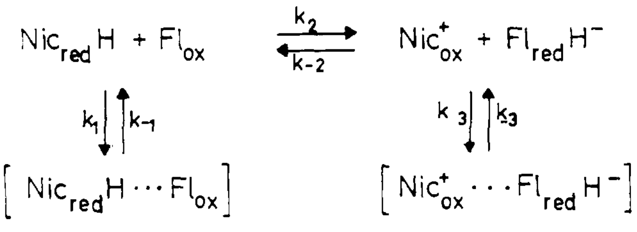

II

Flavine-dependent dihydronicotinamide dehydrogenation can be rationalized by two alternative mechanisms (Scheme I). In mechanism 1 charge transfer complexes are visualized as precursors of oxidoreduction and their formation may thus represent substrate activation. In mechanism 2 charge transfer complexation is visualized as a side reaction which is catalytically not essential. Since charge transfer complexation should be diffusion controlled, the individual rate constants can be estimated: k_1 would have to be in the order of 10^8–10^9 M^{-1} $\sec^{-1}$; with K_d in the order of 0.1 M, k_{-1} would have to be around 10^7–10^8 $\sec^{-1}$. Similar values can be expected for k_3 (10^8 $\sec^{-1}$) and k_{-3} (5×10^8 M^{-1} $\sec^{-1}$). The rate-limiting step involves a rate constant which is at least 10^4 times smaller. Therefore mechanisms 1 and 2 are kinetically indistinguishable. Present evidence supports mechanism 1, although it does not eliminate mechanism 2. This includes the rather low activation energy (8.5 ± 0.5 kcal/mol) of the reaction (Radda and Calvin, 1964; Fox and Tollin, 1966) as well as the observation that steric conditions optimal for charge transfer interactions in flavine-nicotinamide biscoenzymes coincide with maximal oxidoreduction rates (Blankenhorn, 1975). The observed free energy relationship consequently would indicate that the oxidation-reduction potential of free flavine analogs is shifted by a constant factor in the complex with NMNH, demonstrating that the geometry of the complexes investigated does not vary significantly.

Comparison with Biological Results. Comparison between the reaction mechanism suggested for flavine-dependent dihydronicotinamide dehydrogenation in model systems and that of enzymic systems reveals close similarities. Thus in both cases formation of a charge transfer complex occurs prior to oxidoreduction. Its rate of formation has been determined with at least two flavoenzymes (Massey et al., 1970; Matthews and Massey, 1971), however, in model systems it is too fast to be determined by the stopped flow method.

In model systems cleavage of the C(4)–H bond in 1,4-dihydronicotinamide is always rate limiting. In enzyme catalysis other steps may become rate limiting: in several flavoenzymes, dissociation of substrate has been shown to determine the rate of turnover (Massey et al., 1970).

From our model studies one would predict at least two criteria by which the rate of hydrogen transfer from dihydronicotinamide to flavine would be controlled in enzyme catalysis.

(1) *The efficiency of π overlap* between donor and acceptor is controlled by the geometry of the complex. Small changes in distance or angle between the planes of the molecules will cause significant changes in rate (Blankenhorn, 1975). The efficiency of π overlap as well as the rigidity supplied by the protein environment in different enzymes should be reflected in the oscillator strength and molar extinction coefficient of the respective charge transfer transitions. Thus in low molecular weight charge transfer complexes between reduced flavine and oxidized nicotinamide extinction coefficients do not exceed 1000 M^{-1} cm^{-1} (Sakurai and Hosoya, 1966) for intermolecular systems and reaches 1200 M^{-1} cm^{-1} (Proffitt et al., 1974) for an intramolecular system. In enzymic systems, however, values up to 3000 M^{-1} cm^{-1} (Massey and Palmer, 1962) have been observed.

(2) *The difference in oxidation-reduction potential* between donor and acceptor may be changed by the protein environment thereby changing the reaction rate. An obvious way of changing the flavine oxidation-reduction potential is by differential binding of oxidized and reduced form to the protein. Furthermore the acceptor potential may be increased by the proximity of positively charged groups; the donor potential may be increased by negatively charged groups supplied by the protein environment.

Entropy factors also significantly contribute to the reaction rate: for the intermolecular reaction between lumiflavine and NMNH k_2 has a value of 7 $\sec^{-1}$. The corresponding intramolecular reaction, however, is up to 50 times faster (k_2 = 345 $\sec^{-1}$) (Blankenhorn, 1975). The highest turnover number reported for a flavoenzyme, mammalian NADH dehydrogenase, is still 40 times higher (Cremona and Kearney, 1964).

In a protein environment rotational freedom in the flavine-nicotinamide charge transfer complex will be severely restricted. If donor and acceptor became fixed relative to each other, direct hydrogen transfer as observed for the dihydronicotinamide-flavine couple would become stereospecific. In transhydrogenation from NADPH to NAD$^+$, a flavoenzyme-catalyzed reaction, stereospecificity of hydrogen transfer, has been observed (Louie and Kaplan, 1970; Van den Broek and Veeger, 1971). However, enzymes from different sources do not show stereospecificity for the same dehydronicotinamide C(4)–H bond and in some enzymes only stereoselectivity is observed (Louie and Kaplan, 1970). Long wave absorbing intermediates have not been reported for transhydrogenase flavoenzymes as yet.

The Role of Charge Transfer Complexes in Flavoenzyme Catalysis. Charge transfer complexes have been shown to occur in the catalytic cycle of many flavoenzymes (Massey and Ghisla, 1974). Therefore the question arises whether a specific biological function is associated with charge transfer complexation. It has been suggested that donor-acceptor interactions may be importantly contributing to complex stability (Massey and Ghisla, 1974). The results reported in this paper clearly do not support this view. To this author it seems more probable that the intrinsic properties of charge transfer complexes make them fit for participating in catalysis. Thus formation of the complex requires only small activation and is reversible; in the charge transfer complex donor and acceptor are properly oriented for oxidoreduction.

Charge transfer complexes of reduced flavoenzymes might possess another important function: stabilization of the planar antiaromatic dihydroflavine (Massey and Ghisla, 1974; Hemmerich and Schuman-Jörns, 1973). Reactivity of 1,5-dihydroflavine, in particular its oxygen reactivity, has been proposed to depend on the degree of planarity in the molecule (Tauscher et al., 1973).

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Roles of Zinc Ion and Reduced Coenzyme in Horse Liver Alcohol Dehydrogenase Catalysis. The Mechanism of Aldehyde Activation[†]

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ABSTRACT: 1,4,5,6-Tetrahydronicotinamide adenine dinucleotide (H_2NADH) has been investigated as a reduced coenzyme analog in the reaction between *trans*-4-*N,N*-dimethylaminocinnamaldehyde (I) (λ_{max} 398 nm, ϵ_{max} 3.15×10^4 M^{-1} cm^{-1}) and the horse liver alcohol dehydrogenase-NADH complex. These equilibrium binding and temperature-jump kinetic studies establish the following. (i) Substitution of H_2NADH for NADH limits reaction to the reversible formation of a new chromophoric species, λ_{max} 468 nm, ϵ_{max} 5.8×10^4 M^{-1} cm^{-1}. This chromophore is demonstrated to be structurally analogous to the transient intermediate formed during the reaction of I with the enzyme-NADH complex [Dunn, M. F., and Hutchison, J. S. (1973), *Biochemistry 12,* 4882]. (ii) The process of intermediate formation with the enzyme-NADH complex is independent of pH over the range 6.13-10.54. Although studies were limited to the pH range 5.98-8.72, a similar pH independence appears to hold for the H_2NADH system. (iii) Within the ternary complex, I is bound within van der Waal's contact distance of the coenzyme nicotinamide ring. (iv) Formation of the transient intermediate does not involve covalent modification of coenzyme. Based on these findings, we conclude that zinc ion has a Lewis acid function in facilitating the chemical activation of the aldehyde carbonyl for reduction, and that reduced coenzyme plays a noncovalent effector rôle in this substrate activating step.

The use of substrate and/or cofactor analogs to investigate mechanisms of enzyme catalysis often provides an experimental system where individual chemical steps in the overall reaction at the enzyme site can be studied in isolation. This happenstance arises when the free-energy profile for the overall reaction of the analog vis à vis that of natural substrates is substantially altered. Some notable examples which have been fruitfully investigated via this approach include the reaction of α-chymotrypsin with arylacryloyl imidazoles to form acyl-enzymes (Bender and Zerner, 1961; Bernhard et al., 1965), the reaction of glyceraldehyde-3-phosphate dehydrogenase with the pseudosubstrate β-(2-furyl)acryloyl phosphate to form acyl-enzyme intermediate

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