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8-Azidoadenine Analogs of NAD^+ and FAD

Synthesis and Coenzyme Properties with NAD^+-Dependent and FAD-Dependent Enzymes

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The synthesis and purification of the 8-azidoadenine analogs of NAD^+ (azido-NAD^+) and FAD (azido-FAD) from 8-azidoadenosine 5′-phosphate and NMN^+ or FMN, respectively, is described. The coenzyme analogs are characterized by absorption, nuclear magnetic resonance and circular dichroism spectra. The two latter methods indicate a folded structure of azido-NAD^+ and azido-FAD. Upon irradiation at 300 mn in aqueous solution, a change of the ultraviolet absorption spectra of the coenzyme analogs indicates photolysis of the azido group. The coenzyme properties of azido-NAD^+ are demonstrated with lactate, glutamate and alcohol dehydrogenase yielding 14, 154 and 60%, respectively, of the V observed with NAD^+. Concomitantly, the K_m values of the coenzyme analogs are 1.7, 3.5 and 3-fold higher than those of NAD^+. Azido-FAD is shown to be coenzyme of apo-glucose oxidase. The recovery of activity, however, is much slower in the presence of azido-FAD than with FAD. A final value of 66% of the activity with FAD is obtained. With apo-D-amino acid oxidase, azido-FAD is completely inactive, although it is specifically bound to the enzyme.

NAD and FAD play an indispensable role as coenzymes in numerous enzymic oxidation reduction processes. A detailed understanding of the mechanisms of these enzymic reactions is concerned with the investigation of enzyme-coenzyme interactions. One of the main problems in this respect is the characterization of the coenzyme binding site and its localization within the primary structure of the enzyme. Such a chemical approach necessitates a coenzyme analog which specifically binds to the active center and in addition reacts covalently with an adjacent amino acid side chain in order to label this site. For this purpose photoaffinity labeling appears to be the most promising technique, since an appropriate label provides both specificity with respect to the active site and a photolytically generated highly reactive intermediate which is readily stabilized by covalent linkage to a neighbouring group. For example, in the case of an aromatic azide as photolabile species, N_2 is split off upon irradiation yielding a nitrene capable of forming different types of covalent bonds, from the addition to a double bond to a hydrogen abstraction reaction.

Therefore, our aim was to synthesize analogs of NAD^+ and FAD to be used as photoaffinity labels for NAD-dependent and FAD-dependent enzymes. Recently the synthesis of 8-azido analogs of cyclic 3′:5′-AMP [2], 5′-ADP [3] and 5′-ATP [4] has been described. Since these nucleotides have also been successfully used in photoaffinity labeling experiments [3–6], it seemed promising to synthesize the 8-azido-adenine analogs of NAD^+ and FAD. In the present paper the preparation of azido-NAD^+ and azido-FAD from the mononucleotides, 8-azido-AMP and NMN^+ or FMN, respectively, is reported. The nucleotide analogs are characterized and their functional properties as coenzymes are described.

MATERIALS AND METHODS

Reagents

Pig heart lactate dehydrogenase, bovine liver glutamate dehydrogenase, horse liver alcohol dehydrogenase, glucose oxidase from *Penicillium notatum* and pig kidney D-amino acid oxidase were purchased from Boehringer Mannheim GmbH, Germany. All enzymes were used without further purification. The NAD-dependent dehydrogenases were dialyzed for 48 h against three changes of M/15 phosphate buffer pH 7.6 prior to use. The flavoenzymes were converted

This work has been described in a preliminary report [1].

Abbreviations. Azido-NAD^+, nicotinamide 8-azidoadenine dinucleotide; azido-FAD, flavin 8-azidoadenine dinucleotide; CD, circular dichroism; NMR, nuclear magnetic resonance.

Enzymes. Alcohol dehydrogenase or alcohol:NAD^+ oxidoreductase (EC 1.1.1.1); lactate dehydrogenase or lactate:NAD^+ oxidoreductase (EC 1.1.1.27); glutamate dehydrogenase or L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating) (EC 1.4.1.3); glucose oxidase or β-D-glucose:oxygen 1-oxidoreductase (EC 1.1.3.4); D-amino acid oxidase or D-amino-acid:oxygen oxidoreductase (EC 1.4.3.3).

to the apoenzymes according to published procedures [7,8]. AMP, NMN^+, NAD^+, FMN, and FAD were products of Boehringer Mannheim GmbH, Germany. The substrates and all other chemicals were of highest purity grade from commercially available sources. Anhydrous organic solvents were prepared by passage through appropriate columns of activated aluminium oxide.

Preparation of the Nucleotide Analogs

8-Bromo-AMP was prepared by reaction of 10 mmol AMP with a 1.5-molar excess of Br_2 at pH 4.0 under pH-stat conditions. After uptake of one equivalent of bromine, the reaction was stopped by addition of dithionite and the product was purified on a Dowex 1X2 acetate column. Elution with an acetic acid gradient from 0 to 4 M led to a homogeneous main fraction of 8-bromo-AMP, which was lyophilized (yield 90%).

8-Azido-AMP was prepared by heating the tri-*n*-butylammonium salt of 8-bromo-AMP at 70 °C in anhydrous dimethylsulphoxide containing a 5-fold excess of tri-*n*-butylammonium azide. After 6 h the nucleotide was precipitated with diethyl ether and purified on Dowex 1X2 as described for 8-bromo-AMP. The homogeneous main fraction was lyophilized and kept in the dark at 4 °C (yield 65%).

Coupling of 8-azido-AMP with NMN^+ or FMN to yield the desired dinucleotides was performed according to the method of Michelson [9], using diphenylphosphorochloridate as coupling reagent. Azido-NAD^+ was purified on Dowex 1X2 acetate by elution with an acetic acid gradient from 0 to 2 M. The dinucleotide appeared after unreacted NMN^+ and two minor nonidentified fractions and was well separated from bis(8-azidoadenosine) 5′-pyrophosphate and unreacted 8-azido-AMP. Lyophilized azido-NAD^+ (yield 25%) is stable when kept in the dark at 4 °C. Azido-FAD was separated from unreacted mononucleotides on DEAE-Sephadex A-25 chloride using a LiCl gradient from 0 to 50 mM in 10 mM HCl. After this step the dinucleotide was obtained with about 80% purity as estimated from thin-layer chromatography on cellulose. Final purification was achieved by preparative thin-layer chromatography in solvent system 1 on preparative cellulose plates G-1805 from Schleicher & Schüll (Dassel, Germany). The eluted azido-FAD was lyophilized and kept in the freezer (yield 25%).

Analytical Procedures

Thin-layer chromatography was performed on cellulose plastic sheets 1440-LS-254 from Schleicher & Schüll (Dassel, Germany) using the following solvent systems: (1) butanol/glacial acetic acid/water (50/20/30, v/v/v); (2) isobutyric acid/concentrated aqueous ammonia/water (70/1/29, v/v/v); (3) collidine saturated with water. Flavin-containing spots were identified by their fluorescence, nicotinamide-containing spots by blue fluorescence after exposure to butanone/ammonia [10]. Spots absorbing in the ultraviolet showed fluorescence quenching after spraying with fluorescein (0.02% in methanol).

Circular dichroism spectra were measured on a Cary 60 spectropolarimeter equipped with a Cary 6002 CD accessory. Fluorescence spectra were recorded on a Perkin Elmer MPF-3 spectrofluorimeter. Photolysis of the coenzyme analogs was carried out in the excitation compartment of the fluorimeter using a broad band from 300 to 340 nm. Absorption spectra were recorded on a Beckman Acta M VI spectrophotometer. The absorption coefficient of the coenzyme analogs was determined on the basis of the phosphate content according to [11]. NMR spectra were recorded on a Bruker-HFX-90 NMR spectrometer operating in the Fourier transform mode for the dinucleotides, whereas for the mononucleotides the normal (cw) mode was applied.

The enzymic activity of the NAD-dependent dehydrogenases was measured photometrically at 20 °C. At 366 nm the development of NADH was recorded under the following assay conditions. Lactate dehydrogenase: 100 mM lactate, 0.1 – 1 mM NAD^+ or azido-NAD^+ in M/15 phosphate buffer pH 7.6. Glutamate dehydrogenase: 8.5 mM L-glutamate, 0.06 – 0.5 mM NAD^+ or azido-NAD^+ in M/15 phosphate buffer pH 7.6. Alcohol dehydrogenase: 85 mM ethanol, 0.1 – 1 mM NAD^+ or azido-NAD^+ in 0.1 M pyrophosphate buffer pH 8.8. Glucose oxidase activity was measured at 25 °C with an oxygen electrode (Yellow Springs Instrument Co. Ohio, U.S.A.) under the following conditions. Apo-glucose oxidase (0.1 mg/ml) was preincubated at 25 °C with a 10-fold molar excess of FAD or azido-FAD in 0.1 M phosphate buffer pH 6.1. After different time intervals aliquots were assayed for oxygen consumption in the same buffer containing 10 mM glucose and 4 μg/ml catalase. The activity of D-amino acid oxidase was measured at 25 °C under the following conditions. Apoenzyme preincubated with a 10-fold molar excess of FAD or azido-FAD for 1 h was added to a solution of 9.5 mM DL-phenylalanine and 1 μg/ml catalase in 0.1 M pyrophosphate buffer pH 8.5. The formation of phenylpyruvate was measured photometrically at 280 nm.

RESULTS AND DISCUSSION

Synthesis

Analogs of NAD^+ and FAD substituted at the adenine moiety can be obtained by two different

chemical methods, by specific modification of NAD^+ or FAD, respectively, or by coupling of modified adenine nucleotide to nicotinamide or flavin nucleotide. In the case of the 8-azido analogs of NAD^+ and FAD only the latter method is applicable. Direct bromination of the coenzymes would lead to complex mixtures of products. In the case of NAD^+, degradation occurred upon reaction with bromine, a major fraction devoid of nicotinamide was obtained. The flavin moiety, on the other hand, may incorporate bromine into the ring as well as into 8-α-methyl group. In addition, the conversion of the 8-bromo-adenine dinucleotides to the corresponding 8-azido derivatives, which requires heating in an aprotic solvent, would cause cleavage of the pyrophosphate bond, as shown for the reaction of 8-bromo-ADP with azide [3].

Table 1. *Thin-layer chromatography of azido-NAD^+, azido-FAD, the natural coenzymes and the constituent mononucleotides*
R_F values on cellulose observed in solvent systems 1, 2 and 3 as described in Materials and Methods

Nucleotide	R_F value in solvent		
	1	2	3
AMP	30.8	44.1	52.9
Azido-AMP	37.0	50.6	52.9
NAD^+	13.5	37.6	48.0
Azido-NAD^+	19.7	44.1	51.0
NMN^+	28.4	37.6	21.0
FAD	13.5	25.9	68.6
Azido-FAD	21.0	31.1	67.6
FMN	31.0	26.0	57.8

The purified 8-azido analogs of NAD^+ and FAD give single homogeneous spots on thin-layer chromatography. Table 1 shows the R_F values of the synthesized compounds together with those of the constituent mononucleotides in three different solvent systems. The coenzyme analogs show R_F values close to those of the corresponding natural coenzymes but clearly different from those of the constituent mononucleotides.

Absorbance

For further characterization, the absorption spectra of the coenzyme analogs are shown in Fig. 1. In both cases, marked differences are observed between neutral and acidic pH values. These are caused by the azidoadenine moiety, which shows 30% hyperchromicity of the 280-nm absorption upon protonation at N-1 ($pK = 3.9$) and a strong blue shift of the other absorption maximum from 223 nm at neutrality to 215 nm below pH 3 [2,3]. Contrary to NAD^+, the spectrum of azido-NAD^+ clearly reflects typical properties of the nicotinamide absorption. The maximum at 274 nm and the shoulder at 264 nm (Fig. 1 A) are generated by the shoulder (274 nm) and maximum (264 nm) of the absorption of NMN^+ together with the overlapping azidoadenine chromophore ($\lambda_{max} = 282$ nm). The absorption of azido-FAD above 320 nm (Fig. 1 B) is the same as that of natural FAD. Below this wavelength, the spectrum is governed by the strong flavin transition at 264 nm under which the azidoadenine absorption is buried. The presence of this chromophore is best expressed by the ratio of

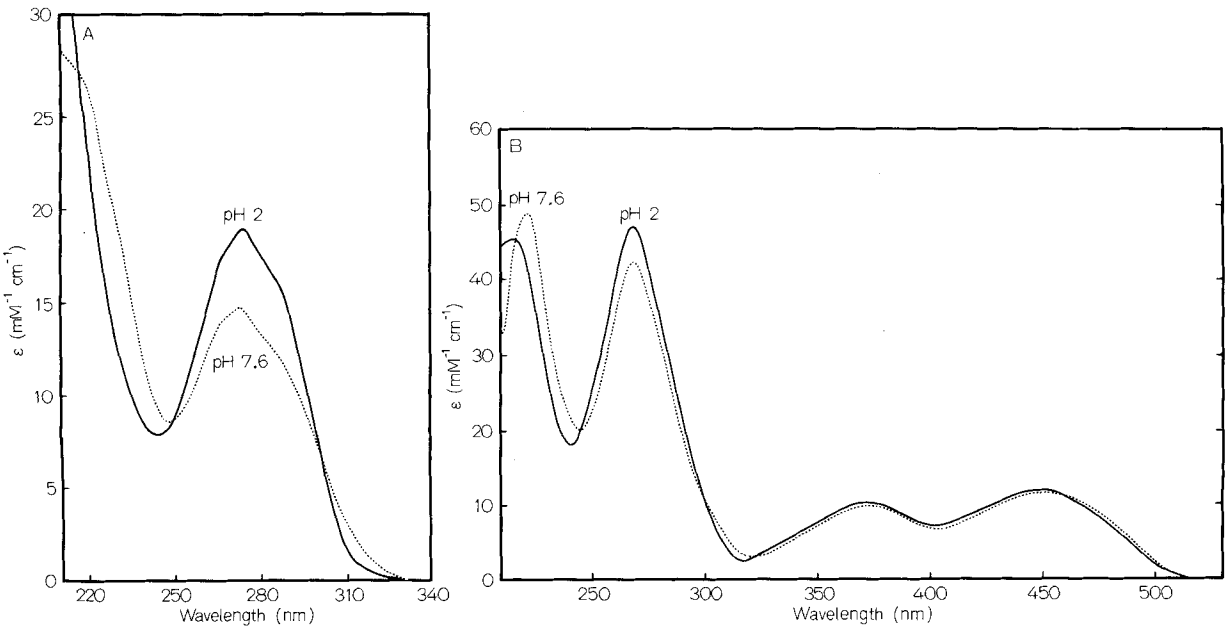

Fig. 1. *Absorption spectra of the synthesized dinucleotides at 20 °C.* (A) Azido-NAD^+; (B) azido-FAD. (— —) pH 2.0, 10 mM HCl; (------) pH 7.6, M/15 phosphate buffer

$\varepsilon_{280}/\varepsilon_{450}$. For pure azido-FAD, a value of 2.80 is obtained at pH 7.6. For comparison, FAD yields $\varepsilon_{280}/\varepsilon_{450} = 1.87$ under the same conditions.

NMR Spectra

The chemical shifts in NMR spectra of azido-NAD^+ and azido-FAD and of the natural coenzymes and the constituent mononucleotides are summarized in Table 2. In addition to the indicated protons, the spectra show only the water band and the ribose and ribityl protons between 5.1 and 4.1 ppm. The NMR data may therefore serve as an additional proof of homogeneity of the synthesized compounds. The chemical shifts obtained for NAD^+, NMN^+ and AMP are identical with published data [12]. In the case of FMN and FAD, however, the chemical shifts reported in the literature vary considerably [12–16]. Moreover, the assignment of the flavin H-9 and H-6 protons is controversial [15]. Our data for FMN and FAD are in good agreement with those of others ([15] and [16], respectively). Comparisons of the data for NAD^+ and azido-NAD^+ (Table 2) shows very close similarity in the chemical shifts of the nicotinamide protons. Their upfield shifts, as compared to NMN^+, clearly reflects the shielding by stacking interaction of the nicotinamide and the adenine ring, suggesting a 'closed' conformation also in the case of azido-NAD^+. In the case of FAD and azido-FAD, very similar chemical shifts are observed for the isoalloxazine protons. Together with the marked upfield shift of the adenine H-2 signal, as compared to AMP, these results indicate similar conformations of FAD and azido-FAD in solution.

Circular Dichroism

Further evidence for 'closed' conformations of azido-NAD^+ and azido-FAD is obtained from CD spectra. As shown in Fig. 2, both nucleotides exhibit complex CD spectra which cannot be expressed by simple addition of the CD spectra of the constituent mononucleotides. The same holds true for the CD spectra of the natural coenzymes NAD^+ and FAD. In detailed CD studies [17,18], these spectra were interpreted on the basis of coupled oscillator theory. Assuming coupling of the transition dipole moments of the adenine with the nicotinamide or the isoalloxazine chromophore to be the main source of rotatory strength, the complex CD spectra could be explained and models for the closed conformation of NAD^+ and FAD were proposed [17,18]. The CD spectra shown in Fig. 2 most likely reflect the same phenomenon of dipole coupling between the participating stacked chromophores. A model of the closed conformation of azido-NAD^+ and azido-FAD, however, cannot be derived, since the orientation of the transition dipole moments of azidoadenine is unknown. The pH dependence of the CD spectra (Fig. 2) mainly reflects the protonation of azidoadenine ($pK = 3.9$) and the concomitant change in the absorption properties of this chromophore (*cf.* Fig. 1). In the case of azido-NAD^+, however, in addition a conformational change is to be expected due to the electrostatic repulsion of the azidoadenine and nicotinamide rings which at pH 2, are positively charged.

Photolysis

In order to demonstrate the photo-lability of the synthesized dinucleotides, irradiation was carried out

Table 2. *NMR chemical shifts of azido-NAD^+, azido-FAD, the natural coenzymes and the constituent mononucleotides*
Measurements in ^2H_2O, pH 7, 23 ± 2 °C; mononucleotides 30 mM, dinucleotides 5 mM; chemical shifts measured from 2,2-dimethyl-2-silapentane-5-sulfonate

Atom or group	Chemical shift in							
	AMP	azido-AMP	FMN	FAD	azido-FAD	NMN^+	NAD^+	azido-NAD^+
	ppm							
Adenine H-8	8.50			8.32			8.41	
Adenine H-2	8.16	8.08		7.87	7.77		8.12	8.08
Adenine H-1′	6.10	5.94		5.88	5.73		6.06	5.84
Nicotinamide H-6						9.35	9.16	9.12
Nicotinamide H-5						8.31	8.19	8.16
Nicotinamide H-4						9.01	8.84	8.80
Nicotinamide H-2						9.58	9.34	9.29
Nicotinamide H-1′						6.21	6.06	6.04
Flavin H-9			7.37	7.55	7.62			
Flavin H-6			7.67	7.60	7.67			
Flavin CH_3-8			2.45	2.42	2.49			
Flavin CH_3-7			2.29	2.35	2.39			

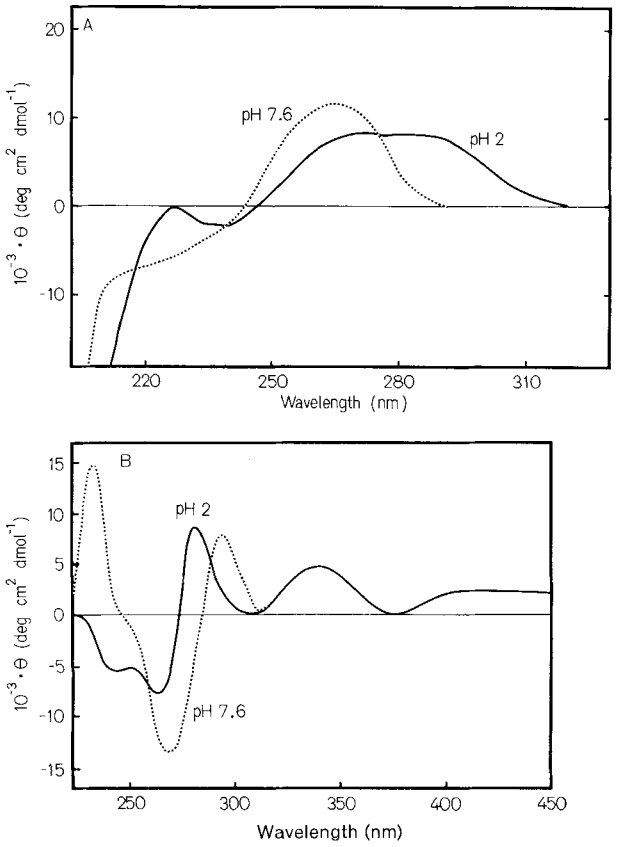

Fig. 2. *Circular dichroism spectra of the synthesized dinucleotides at 20 °C.* (A) Azido-NAD$^+$; (B) azido-FAD. (——) pH 2.0; (- - -) pH 7.6; solvents as in Fig. 1

followed by measurement of the absorption spectra. Fig. 3 shows that both azido-NAD$^+$ and azido-FAD change their absorption after irradiation at 300 nm due to degradation of the azidoadenine chromophore. This has been confirmed by a control experiment under the same conditions with 8-azido-AMP which leads to a similar pattern: the 282-nm absorption of the azidoadenine disappears upon irradiation and a new maximum at 274 nm is generated [3]. The mechanism of such a photolysis can be described according to Knowles [19] to occur in two steps as follows. In a first light-dependent reaction, molecular nitrogen is split off generating a short-lived highly reactive nitrene. This is stabilized in a second step by either intramolecular rearrangement or covalent reaction with a surrounding group. In the case of 8-azido-adenine nucleotides the intermolecular mechanism seems to predominate, since 8-azido-3′:5′-AMP [5, 6], 8-azido-ATP [4], and 8-azido-ADP [3] have been successfully used as photoaffinity labels. Thus, the product obtained after photolysis of 8-azido-AMP in water, is expected to be the 8-hydroxylamino derivative. This, however, needs to be established by independent synthesis.

Coenzyme Properties

The biological activity of the synthesized dinucleotides was tested by their ability to function as coen-

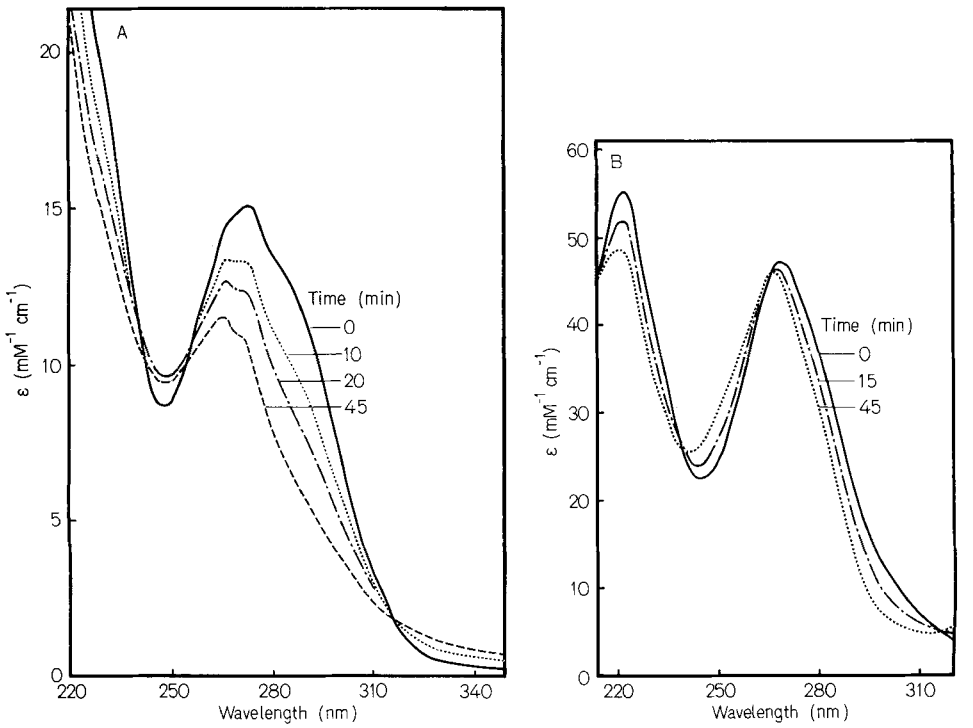

Fig. 3. *Change of the absorption spectra of the synthesized dinucleotides after irradiation.* Samples in M/15 phosphate buffer pH 7.6 were irradiated for the indicated time periods at 300 nm and the spectra were taken. (A) Azido-NAD$^+$; (B) azido-FAD

Table 3. *Enzymic activity of NAD$^+$-dependent and FAD-dependent enzymes with azido-NAD$^+$ and azido-FAD*
Measurements were made under the conditions given in Materials and Methods; V', K'_m are maximum velocity and Michaelis constant in the presence of coenzyme analog; V, K_m are maximum velocity and Michaelis constant in the presence of natural coenzyme under the same conditions

Enzyme	K_m	K'_m	V'/V
	mM		
Lactate dehydrogenase	0.074	0.125	0.14
Glutamate dehydrogenase	0.14	0.50	1.54
Alcohol dehydrogenase	0.065	0.19	0.60
Glucose oxidase	–	–	0.66[a]
D-Amino acid oxidase	–	–	0

[a] This value represents the maximum recovery of activity of apo-glucose oxidase with azido-FAD after 24 h as compared to FAD.

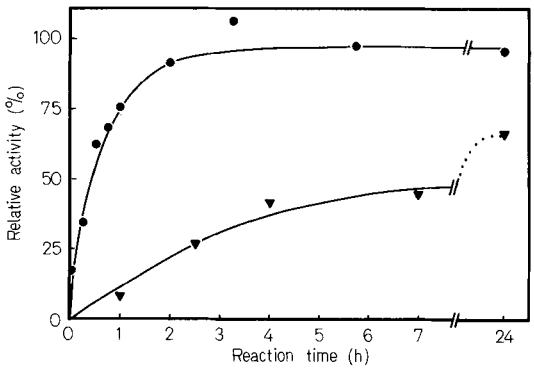

Fig. 4. *Reactivation of apo-glucose oxidase with FAD and azido-FAD.* Apo-glucose oxidase (0.1 mg/ml) was incubated with a 10-fold molar excess of FAD or azido-FAD in 0.1 M phosphate buffer pH 6.1 at 25 °C. After different incubation times the enzymic activity was assayed with an oxygen electrode as described in Materials and Methods. (●) Reactivation with FAD; (▼) reactivation with azido-FAD. Maximum reactivation with FAD corresponds to about 80% of the initial activity of holo-glucose oxidase

zymes with NAD-dependent and FAD-dependent enzymes. Table 3 shows the K_m values of azido-NAD$^+$ and NAD$^+$ with three different dehydrogenases as well as the relative maximum velocities. In all cases, the Michaelis constants of azido-NAD$^+$ are increased (1.7 – 3.5-fold) relative to those of NAD$^+$. Surprisingly, in the case of glutamate dehydrogenase a 1.5-fold higher maximum velocity is observed with azido-NAD$^+$ than with NAD$^+$. It should be remembered, however, that this value is extrapolated from intermediate NAD$^+$ concentrations. At higher NAD$^+$ concentrations, glutamate dehydrogenase exhibits coenzyme self-activation [20], leading to K_m and V values [21] identical to those obtained with azido-NAD$^+$. The same behaviour has recently been found with glutamate dehydrogenase and the 1:N^6-etheno derivative of NAD$^+$ [21] suggesting that the active site of glutamate dehydrogenase displays only a little specificity toward the intact adenine moiety of the coenzyme. With alcohol and lactate dehydrogenase the V for azido-NAD$^+$ is only 60% and 14%, respectively, of the NAD$^+$ reduction rate indicating different sensitivity of the three dehydrogenases with respect to modification of the adenine moiety. The reaction with alcohol dehydrogenase has been used to prepare a small amount of azido-NADH which was purified on DEAE-cellulose according to Gurr *et al.* [22]. The reduced coenzyme analog shows the typical dihydronicotinamide absorption at 340 nm in addition to the 282-nm absorption of the azido-adenine moiety. The reduced form, however, is stable only for several hours even at pH 9, as judged from the decrease of the 340-nm band.

Azido-FAD is a coenzyme for glucose oxidase. When incubated with azido-FAD, apo-glucose oxidase exhibits a final value of 66% of the enzymic activity obtained after incubation with natural FAD (Table 3). The time course of reactivation, however, is different for the two coenzymes (Fig. 4). With FAD, maximum recovery of glucose oxidase is reached after about 120 min whereas in the case of azido-FAD activity still increases after 6 h reaching equilibrium after about 24 h. This indicates that azido-FAD is less effective than FAD as initiator of the reactivation process of the enzyme. With D-amino acid oxidase, azido-FAD is completely inactive as coenzyme (Table 3), even after prolonged incubation. Under these conditions, FAD restores enzymic activity of apo-D-amino acid oxidase completely within 15 min. Similar differences between glucose oxidase and D-amino acid oxidase have been reported with respect to the enzymic activity with the coenzyme analog flavin – 1:N^6-ethanoadenine dinucleotide [23].

Although completely inactive as coenzyme, azido-FAD specifically binds to D-amino acid oxidase as indicated by the difference spectra shown in Fig. 5. For the enzyme · azido-FAD complex a characteristic blue shift centered at 493 nm is observed. In contrast to this, a red shift at the same wavelength has been reported for the enzyme · FAD complex [8] which has been confirmed in our laboratory. This may indicate different environments of the isoalloxazine moiety of FAD and azido-FAD in the binary complexes. The difference spectrum of the ternary enzyme · azido-FAD · benzoate complex (Fig. 5) is identical to that obtained with FAD [24] instead of azido-FAD, suggesting close similarity of both ternary complexes. The affinity of apo-D-amino acid oxidase for azido-FAD is much lower than for FAD as judged from gel-filtration experiments on Sephadex G-25 yielding complete separation of apoenzyme and azido-FAD even in the presence of benzoate. Under these

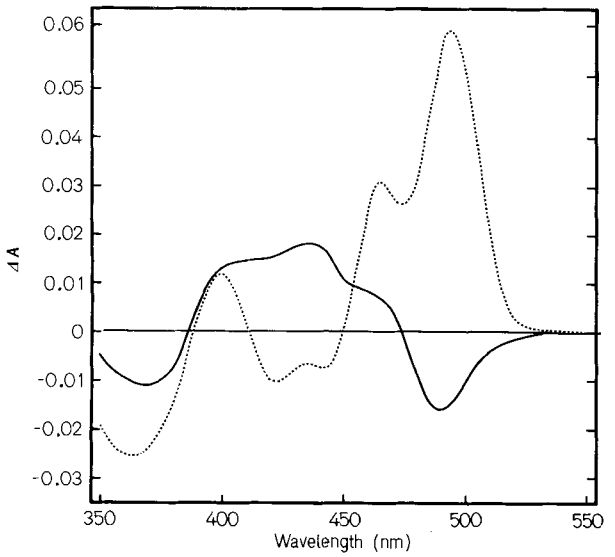

Fig. 5. *Absorption difference spectra of D-amino acid oxidase complexes with azido-FAD.* Measurements in 0.1 M pyrophosphate buffer pH 8.5 at 20 °C. Reference cuvette: 140 μM azido-FAD. Sample cuvette: 140 μM azido-FAD, 22 μM apo-D-amino acid oxidase; (–) without benzoate; (·····) with 1 mM benzoate. The spectra are corrected for a slight absorption of the apoenzyme at 350–430 nm

conditions FAD is not removable from the holoenzyme. Quantitative binding studies of azido-FAD to D-amino acid oxidase are hampered by the instability of the apoenzyme under the conditions of equilibrium dialysis and difference spectroscopic titration. Therefore, from difference spectra in the presence of benzoate, the dissociation constant of azido-FAD could only be estimated to be below 10^{-5} M.

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