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NAD⁺ and NAD⁺ analogues in horse liver alcohol dehydrogenase. Relationship between reactivity and conformation simulated with molecular mechanics

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In the present study we show that the enzymatic activity of the coenzyme nicotinamide adenine dinucleotide (NAD⁺) and its analogues (C(O)NH₂ replaced by C(S)NH₂, C(O)CH₃, C(O)H and CN) with horse liver alcohol dehydrogenase (LADH) (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) can be rationalized by their conformation in the active site determined with molecular mechanics (AMBER, assisted model building with energy refinement). In order to establish the relation between the hydride transfer rate and the conformation of the NAD⁺ and its analogues, kinetic experiments with the poor substrate isopropanol were carried out. It appears that the enzymatic activity can be readily explained by the geometry of the pyridinium ring, in particular the magnitude of the 'out-of-plane' rotation of the carboxamide side chain (or analogues). The latter is nicely illustrated in the case of 3-cyanopyridine adenine dinucleotide which lacks any 'out-of-plane' rotation and concomitantly exhibits no significant enzymatic activity.

Introduction

More insight into the mechanism of dehydrogenase action can be gained by examining the effects of replacing the coenzyme NAD⁺ by NAD⁺ analogues. Of special interest are those which are modified in the reactive part, the nicotinamide moiety. Kinetic data with such analogues have been published on various dehydrogenases [1–11], but no systematic explanation has so far been provided for their reactivities. In the present paper a qualitative explanation using molecular mechanics calculations is reported.

Horse liver alcohol dehydrogenase (LADH) (alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) has been

chosen for the present study because much is known about this enzyme, particularly the X-ray structure of a ternary complex with Me₂SO [12]. Furthermore, the enzyme shows a broad specificity and is therefore of great potential utility in preparative applications.

The chemical step in the enzymatic conversion is the hydride ion transfer from the alcohol to the coenzyme or vice versa. If NAD⁺ is used as coenzyme and ethanol as substrate, the rate-limiting step is the dissociation of the coenzyme from the enzyme [13]. On the other hand, when the poor substrate isopropanol is used, it has been shown by presteady-state measurements that the hydride transfer is rate limiting [13]. In order to relate the hydride transfer rate to the conformation of the NAD⁺ analogues in the active site, kinetic experiments with isopropanol were therefore carried out in this study. It is self-evident, that, for comparing various analogues, the intrinsic reactivity of each, i.e., the non-enzymatic reaction rate with dithionite, must also be taken into account.

Referring to the required structural data, the position of the pyridinium moiety relative to the substrate should first be studied. There is, however, a second structural factor that may be involved: the 'out-of-plane' rotation ϕ of the side chain of the pyridinium ring (Fig. 1a).

The concept of the 'out-of-plane' rotation has been introduced by Donkersloot and Buck [14]. According to

Abbreviations: LADH, horse liver alcohol dehydrogenase; Me₂SO, dimethyl sulfoxide; NAD⁺/NADH, nicotinamide adenine dinucleotide and its reduced form; ac³PdAD⁺/ac³PdADH, 3-acetylpyridine adenine dinucleotide and its reduced form; sNAD⁺/sNADH, thionicotinamide adenine dinucleotide and its reduced form; fPdAD⁺/fPdADH, 3-formylpyridine adenine dinucleotide and its reduced form; cn³PdAD⁺/cn³PdADH, 3-cyanopyridine adenine dinucleotide and its reduced form; UV/VIS, ultraviolet/visible light; AMBER, assisted model building with energy refinement; MNDO, minor neglect of differential overlap program.

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Fig. 1. (a) 'Out-of-plane' rotation of the carboxamide side chain, $\phi = 0$: The CO group is situated in the plane of the pyridinium ring (in the orientation drawn), (b) and (c) represent the entry of the hydride ion in the direction of the carbonyl group. (b) A specificity; (c) B specificity.

their quantum chemical calculations, the enthalpy of the transition state of the hydride transfer is lowered when the carboxamide group is rotated out of the pyridinium plane, the carbonyl group pointing towards the face of the plane to which the hydride ion is transferred.

The calculations were experimentally verified with model compounds having the CO group forced out of the pyridinium plane [15,16]. The rate of transfer to the face of the plane where the carbonyl group protrudes proved to exceed the rate at the opposite face to such an extent as to produce complete stereospecificity in the absence of any enzyme: A specificity in the case of Fig. 1b, B specificity in Fig. 1c.

In dehydrogenases the A and B specificity is determined by the spatial relationship of substrate and pyridinium moiety (A specificity in LADH, B specificity in GAPDH). The 'out-of-plane' rotation will therefore affect, not the specificity, but the rate of hydride transfer; for effective enzyme action the side chain carbonyl group should therefore be directed more or less towards the substrate. Such is indeed the case in LADH ($\phi = 30^\circ$, [12]) and in GAPDH ($\phi = 22^\circ$, [17]), as observed by X-ray analysis.

For the present study we used the analogues thionicotinamide adenine dinucleotide (sNAD⁺), 3-acetylpyridine adenine dinucleotide (ac³PdAD⁺), 3-formylpyridine adenine dinucleotide (fPdAD⁺) and 3-

cyanopyridine adenine dinucleotide (cn³PdAD⁺) carrying the pyridine side chains C(S)NH₂, C(O)CH₃, C(O)H and CN, respectively. Unfortunately, for the corresponding LADH-coenzyme complexes no X-ray data are available. We therefore resorted to molecular mechanics calculation to assess the geometry of the analogues and its interactions with the enzyme. This procedure proved to be reliable for NAD⁺ as the calculational results were found to correlate closely with the available X-ray data [18,19].

It will be shown in the present paper that the enzymatic activity of the NAD⁺ and its analogues can be rationalized in terms of geometric features, taking into account the intrinsic reactivities.

Materials and Methods

Enzyme kinetics

Horse liver alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer-Mannheim. NAD⁺, ac³PdAD⁺, sNAD⁺ and fPdAD⁺ were obtained from Sigma Chemical Company. The substrates ethanol and isopropanol, and the phosphates of the 0.047 M phosphate buffer used (with 0.25 mM EDTA, pH = 7.0, $\Gamma = 0.1$) were from Merck. The purity of the alcohols was checked using gas chromatography. Enzyme solutions were centrifuged prior to use. Kinetic experiments were carried out at 22°C on a Hitachi 150-20 UV/VIS spectrophotometer equipped with a data processor. The steady-state initial rates of NAD⁺, sNAD⁺, fPdAD⁺ and ac³PdAD⁺, respectively, were measured following the formation of the reduced pyridine nucleotide at an appropriate wavelength (NAD⁺/NADH, $\lambda = 340$ nm, $\epsilon = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$; sNAD⁺/sNADH, $\lambda = 396$ nm, $\epsilon = 10000 \text{ M}^{-1} \cdot \text{cm}^{-1}$; ac³PdAD⁺/ac³PdADH, $\lambda = 363$ nm, $\epsilon = 9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$; fPdAD⁺/fPdADH, $\lambda = 358$ nm, $\epsilon = 7800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [8,20,21]). The concentration range of the coenzyme was 10–100 μM (with the exception of fPdAD⁺, which varied from 100–1000 μM). The substrate concentration ranged from 2–19 mM.

Kinetic parameters in the initial-rate equation [13]:

$$1/V = e/k_{\text{cat}}(1 + K_a/[A] + K_b/[B] + K_c/[A][B]) \quad (1)$$

were obtained from the primary and secondary plots of the initial-rate data. In Eqn. 1, e is the concentration of enzyme active centres (twice the molar concentration); A and B represent coenzyme and substrate, respectively; K_a , K_b and K_c denote the kinetic coefficients.

At saturating ethanol concentration the measured k_{cat} values (data not shown) are in accordance with literature data [6,7,9–11,22,23].

Procedure for calculational studies

Energy calculations and total energy minimization energies were performed with the AMBER molecular

mechanics package (version 3.0) [24] on a VAX 11/785 computer. We used AMBER to minimize a total energy function consisting of separate terms covering bond-stretching (I), -bending (II) and torsional angles (III), as well as Van der Waals, electrostatic (IV) and hydrogen bond (V) interactions (AMBER energies, Eqn. 2).

$$E_{\text{total}} = \sum_{\text{bonds}} K_R (R - R_{\text{eq}})^2 + \sum_{\text{angles}} K_\theta (\theta - \theta_{\text{eq}})^2 + \sum_{\text{dihedrals}} \frac{V_n}{2} [1 + \cos(n\phi - \gamma)] + \sum_{i < j} \left(\frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^6} + \frac{q_i q_j}{\epsilon R_{ij}} \right) + \sum_{\text{H bonds}} \left(\frac{C_{ij}}{R_{ij}^{12}} - \frac{D_{ij}}{R_{ij}^{10}} \right) \quad (2)$$

The procedure is identical to the method described in our previous papers [18,19]. The active site of LADH was constructed from X-ray crystallographic data of a ternary complex of NAD⁺/LADH/Me₂SO reported by Eklund and co-workers [12] (0.29 nm resolution, crystallographic *R* factor of 0.22) which was readily retrievable from the Brookhaven Protein Database. The three-dimensional structures of the NAD⁺ analogues were derived directly from the X-ray NAD⁺ geometry using an interactive computer graphics program (Chem-X, January 1989 update, copyright Chemical Design Oxford, Oxford). During the energy minimization the amino acids and the zinc ion were fixed at their initial positions. The cysteine residues of the core of amino acids (Cys-46 and Cys-174) were introduced as negatively charged residues as described in our earlier paper [18]. All minimizations were performed until the RMS gradient value of the energy was less than 4.18 kJ · nm⁻¹ (1.0 kcal · nm⁻¹), using the distance-dependent dielectric constant and treating all CH, CH₂ and CH₃ groups as united atoms.

Atomic charges were calculated using the MNDO semi-empirical molecular orbital method. Most harmonic force constants were obtained from the literature or extrapolated from available data [25,26]. For the calculation of the 'out-of-plane' rotation appropriate values for the rotation barriers *V_n* had to be inserted in Eqn. 2. However, for the pyridinium compounds no experimental values were found in literature, and moreover quantum chemical calculations did not provide sufficiently reliable results [14,27]. The experimental values of the closely related benzene derivatives, benzamide, benzaldehyde and benzophenone [28,29] were therefore taken. These values may be assumed to be a close approximation to those of the pyridinium compounds NAD⁺ (sNAD⁺), fPdAD⁺ and ac³PdAD⁺, respectively, as benzaldehyde has the same rotation barrier as pyridine-3-aldehyde, 4.7 and 4.6 kcal/mol, respectively [29]. The

TABLE I

Parameters for the 'out-of-plane' torsion angle of NAD⁺ and its analogues

Compound	<i>V_n</i> /2 (kcal · mol ⁻¹)	γ (deg)	<i>n</i>
NAD ⁺	0.0	180	2
sNAD ⁺	0.0	180	2
ac ³ PdAD ⁺	1.55	180	2
fPdAD ⁺	2.35	180	2

rotation barrier was neglected for NAD⁺ and sNAD⁺, since in benzamide a mean 'out-of-plane' rotation of 39° ± 2° was observed [30], i.e., very close to the 45° to be observed when there is no rotation barrier at all. * The torsion potentials introduced for the coenzymes are summarized in table I.

Results and Discussion

Molecular mechanics calculations

The geometries of the energy minimized sNAD⁺, ac³PdAD⁺, fPdAD⁺ and cn³PdAD⁺, with respect to the energy minimized refined NAD⁺ geometry, are given in Fig. 2. Since conformational differences (Table II) are restricted to the nicotinamide (or analogues) moiety, only these regions of the structures are drawn. Neither the zinc ion, nor the amino acids are depicted since they are invariant during all calculations.

As shown previously [18,19], it appears that with molecular mechanics calculations there is a correct positioning of the NAD⁺ pyridinium ring. From the present results, it is also clear that the calculated 'out-of-plane' torsion angle of NAD⁺ (34°) fits well with the X-ray value (30°) reported by Eklund et al. [12] (see Table II). This further supports the correctness of our molecular mechanics approach.

Fig. 2 shows that in contrast to sNAD⁺ the position of the pyridinium rings of ac³PdAD⁺, cn³PdAD⁺ and fPdAD⁺ deviate from the NAD⁺ pyridinium ring. It can also be observed that fPdAD⁺ exhibits an opposite deviation as compared to ac³PdAD⁺ and cn³PdAD⁺.

As far as the 'out-of-plane' torsion angle is concerned the thiocarbonyl group of the energy minimized sNAD⁺ structure shows a larger torsion angle (47°) than the carbonyl group of NAD⁺ (table II), while the torsion angles of ac³PdAD⁺ and fPdAD⁺ are strongly

* The difference in the introduced rotation barrier between the aldehyde and the ketone on the one hand and the amide on the other hand can be easily understood. The carboxamide group have their own resonance stabilization and therefore have no tendency to resonance interaction with the aromatic ring, whereas the aldehyde and ketone groups will do so. Their interactions will stabilize the flat conformation and increase the barrier.

3 contd.

2

Fig. 2. Stereodiagrams of the nicotinamide moiety of the optimized NAD^+ analogue geometries (—). The structure represented by the broken line is the energy refined geometry of NAD^+ ; (a) sNAD^+ ; (b) ac^3PdAD^+ ; (c) fPdAD^+ ; and (d) cn^3PdAD^+ .

Fig. 3. Stereodiagrams of the position of the nicotinamide moiety of the optimized NAD^+ derivatives towards the main chain NH group of Phe-319 and the SO group of the substrate Me_2SO . (a) NAD^+ ; (b) sNAD^+ ; (c) ac^3PdAD^+ ; (d) fPdAD^+ ; and (e) cn^3PdAD^+ .

TABLE II

Conformational parameters of energy-refined NAD^+ and NAD^+ derivatives

Nomenclature of the coenzyme (analogue) torsion angles are the same as those used before [19].

Compound	Torsion angle in degrees										out-of-plane ϕ
	χ_A	γ_A	β_A	α_A	ζ_A	ζ_N	α_N	β_N	γ_N	χ_N	
NAD^+	253	292	153	65	91	201	57	185	53	271	34
sNAD^+	253	292	154	64	90	202	56	187	55	270	47
ac^3PdAD^+	253	292	153	65	90	201	57	185	55	272	6.5
fPdAD^+	253	292	153	65	90	203	56	191	50	274	9
cn^3PdAD^+	253	291	153	65	91	201	57	186	55	272	–

diminished (6.5 and 9° , respectively). Obviously, cn^3PdAD^+ exhibits no 'out-of-plane' rotation at all.

This is further illustrated in Fig. 3, which outlines the position of the nicotinamide (or analogue) moiety of the coenzyme with respect to the main chain NH group of Phe-319 and the substrate Me_2SO . Relevant data are given in Table III. Particularly the long distance between the carbonyl oxygen of fPdAD^+ and the sulfur atom of Me_2SO is noteworthy, as will be discussed later.

Kinetic studies

Table IV summarizes our steady-state kinetic data obtained for NAD^+ and its analogues with isopropanol as substrate, in which the actual hydride transfer becomes rate limiting (see Introduction).

It appears that sNAD^+ and ac^3PdAD^+ are more active than NAD^+ , 4.1- and 2.3-times, respectively, whereas fPdAD^+ is less active (i.e. approx. 10% of NAD^+). Finally, it is shown that cn^3PdAD^+ is completely inactive.

In order to compare the kinetic data summarized in Table IV, the intrinsic reactivities of the compounds, $k_{\text{SO}_2^-}$, as measured by the addition of dithionite anion must also be taken into account (Table V).

TABLE III

Geometric and kinetic data of energy-refined NAD^+ and NAD^+ derivatives

Compound	ϕ (deg)	N(Phe 319)- O(C(O)R) (nm)	S(Me_2SO)- O(C(O)R) (nm)	C4(analog)- C4(NAD^+) ^a (nm)	k_{cat} ^b (rel)
NAD^+	34(30 $^\circ$)	0.29 ± 0.02	0.42 ± 0.02	–	1
sNAD^+	47	0.34^d	0.39^d	0.02	4.12
ac^3PdAD^+	6.5	0.29	0.44	0.04	2.35
fPdAD^+	9	0.29	0.51	0.06	0.12
cn^3PdAD^+	–	–	–	0.04	< 0.01

^a The C4 distance between NAD^+ and the analogue is a measure for the pyridinium shift.

^b k_{cat} values are from Table IV.

^c Value obtained from X-ray analysis [12].

^d Instead of oxygen, sNAD^+ has a sulfur atom bound in the side chain of the pyridinium ring.

Discussion

The reported results permit a correlation to be made between the geometric features of NAD^+ and its analogues in the active site and the measured kinetic data with isopropanol, taking into account the intrinsic reactivities.

TABLE IV

Kinetic parameters derived from Eqn. 1 for LADH with NAD^+ , ac^3PdAD^+ , sNAD^+ , fPdAD^+ , cn^3PdAD^+ and the substrate isopropanol

Compound	Isopropanol		
	K_a (μM)	K_b (mM)	k_{cat} (%)
NAD^+	37	1.3	100 ^a
sNAD^+	249	6.0	412
ac^3PdAD^+	309	3.5	235
fPdAD^+	261	0.5	12
cn^3PdAD^+	–	–	< 1 ^b

^a Equals 0.85 s^{-1} , in accordance with literature data [13].

^b See Refs. 31 and 32.

TABLE V

Kinetic data of NAD^+ and derivatives in the reaction with dithionite and in the enzymatic reduction with isopropanol

Compound	k_{cat} (isoprop) (rel)	$k_{\text{SO}_2^-}$ (rel)	ϕ (deg)
NAD^+	1.0	1 ^a	34
sNAD^+	4.12	3 ^a	47
ac^3PdAD^+	2.35	22 ^a	6.5
fPdAD^+	0.12	16 ^b	9
cn^3PdAD^+	< 0.01	32 ^b	–

^a NAD^+ , $47 \text{ M}^{-1} \cdot \text{s}^{-1}$; ac^3PdAD^+ , $1050 \text{ M}^{-1} \cdot \text{s}^{-1}$; and sNAD^+ , $150 \text{ M}^{-1} \cdot \text{s}^{-1}$. (see Ref. 33).

^b The intrinsic reactivity is calculated from the known linear relationship between the $\log k_{\text{SO}_2^-}$ (rate constant) for the dithionite reduction and the redox potential, showing a higher rate of dithionite reduction with increasing potential [33]. With a redox potential of -262 mV [34], for fPdAD^+ an intrinsic reactivity of about $730 \text{ M}^{-1} \cdot \text{s}^{-1}$ has been obtained. Similarly a redox potential of -240 mV [31] for cn^3PdAD^+ results in a $k_{\text{SO}_2^-}$ of $1513 \text{ M}^{-1} \cdot \text{s}^{-1}$ [33].

Fig. 4. Stereodiagram showing the pyridinium positions of ac^3PdAD^+ (—) and fPdAD^+ (-----).

The high k_{cat} value of sNAD^+ compared to NAD^+ , exhibiting identical positions of their pyridinium rings, can easily be explained by the large value for the 'out-of-plane' orientation and the high intrinsic reactivity (see Table V).

Although the intrinsic reactivity of ac^3PdAD^+ is higher than that of sNAD^+ (Table V) its enzymatic activity is lower. Since the position of the pyridinium ring of ac^3PdAD^+ only slightly differs from NAD^+ , this observation must be mainly explained by the low 'out-of-plane' torsion angle of the acetyl group.

The intrinsic reactivity of fPdAD^+ is almost equal to that of ac^3PdAD^+ , as are their 'out-of-plane' torsion angles. One should therefore expect about equal activities in the enzyme. Actually the activity of fPdAD^+ is lower by an order of magnitude (Table IV). This might be due to the position of its head group not being precisely maintained, as a consequence of the lack of the methyl group (or the NH_2 group) in the side chain; the difference between C4 (ac^3PdAD^+) and C4 (fPdAD^+) is 0.09 nm. This is also indicated by the long distance between the carbonyl oxygen and the sulfur atom of Me_2SO , corresponding to the substrate carbon atom that donates the hydride ion (Fig. 3 and Table III).

Most striking are the data obtained with the cyano analogue. According to our calculations the position of the pyridinium ring of cn^3PdAD^+ virtually coincides with that of the pyridinium ring of ac^3PdAD^+ . However, the cyano analogue is practically inactive in the dehydrogenase, notwithstanding its high intrinsic reactivity. The conclusion can be drawn that this is due to the absence of any 'out-of-plane' rotation of the CN group (vide infra).

The enhancing effect of the carboxamide side chain is ascribed to the δ^- charge of the carbonyl oxygen (-0.28 e.u.), stabilizing the positive charge on C1 of the substrate in the transition state [15]. In the case of the cyano analogue the cyano group occupies an unfavourable position (Fig. 3), the distance between the δ^- charge of the nitrogen atom and the sulphur atom of the substrate (0.54 nm) being larger than the distance between the δ^+ charge of the carbon atom and the sulphur atom (0.48 nm). The cyano group may therefore even retard the hydride transfer.

In conclusion, we have demonstrated that the kinetic data obtained with the NAD^+ analogues can be related to their geometry in the alcohol dehydrogenase. Particu-

larly the magnitude of the 'out-of-plane' rotation of the side chain of the pyridinium ring is decisive.

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