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Kinetic and modelling studies of NAD^+ and poly(ethylene glycol)-bound NAD^+ in horse liver alcohol dehydrogenase

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Abstract

Poly(ethylene glycol)-bound nicotinamide adenine dinucleotide (PEG- NAD^+) has been successfully employed in the continuous production of L-amino acids from the corresponding α -keto acids by stereospecific reductive amination. Like many other dehydrogenases also horse liver alcohol dehydrogenase (HLADH) appears to be active with PEG- NAD^+ as coenzyme, although the turnover number is three to four times lower. The possibilities were considered that the PEG-tail of a PEG- NAD^+ bound to one active site of the HLADH dimer prevents the binding of another PEG- NAD^+ to the second site, or that the PEG-tail causes destabilization of the active dimer. Both could be ruled out by kinetic studies. Neither can the observed lower intrinsic reactivity of PEG- NAD^+ account for the diminished activity of the enzyme. Molecular dynamics studies, on the other hand, show that the pulling action of the polymer chain shifts the NAD position in the active site in the outside direction, causing small but significant changes in the enzyme/coenzyme interactions of a sufficient extent to explain the experimental results.

Keywords: Poly(ethylene glycol)-bound NAD^+ ; Alcohol dehydrogenase; Kinetic modelling; Reductive amination, stereospecific; Molecular dynamics and mechanics; (Horse liver)

1. Introduction

A number of synthetically useful enzymatic reactions require cofactors such as nicotinamide adenine dinucleotide (NAD^+). These cofactors are too expensive to be used as stoichiometric reagents in commercial applications. Regeneration of the cofactors from their reaction by-products is thus required to make the process economical. Many procedures have been suggested to achieve efficient coenzyme recycling, including enzymatic and non-enzymatic methods [1]. One of the systems investigated, the enzyme

membrane reactor concept developed by Wandrey and co-workers [2,3], shows commercially attractive features. For example, continuous production of L-amino acids from the corresponding α -keto acids by stereospecific reductive amination has been achieved with little cofactor consumption in a membrane reactor in which NAD^+ is covalently linked to poly(ethylene glycol) (PEG- NAD^+ , Fig. 1) [4].

Many other dehydrogenases accept the PEG- NAD^+ as coenzyme. So far, glucose dehydrogenase is the only enzyme known that is not active with PEG- NAD^+ [5]. Until now, the observed activities of PEG- NAD^+ with dehydrogenases are not yet understood at the molecular level.

The NAD^+ / NADH dependent horse liver alcohol dehydrogenase (HLADH), which stereoselectively catalyses the interconversions of alcohols to their corresponding aldehyde/ketones and vice versa, seems an ideal candidate for studies aiming at the elucidation of coenzyme/apo-enzyme interactions. Many kinetic data and results from X-ray crystallographic studies on binary and ternary complexes are available for HLADH [6–13], making this enzyme one of the best characterized dehydrogenases.

Abbreviations: NAD^+ , nicotinamide adenine dinucleotide (oxidized form); NADH , nicotinamide adenine dinucleotide (reduced form); PEG- NAD^+ , poly(ethylene glycol)-bound nicotinamide adenine dinucleotide (oxidized form); PEG- NADH , reduced form of PEG- NAD^+ ; NMN^+ , nicotinamide mononucleotide; MM, molecular mechanics; MD, molecular dynamics; AMBER, assisted model building with energy refinement; PEG, poly(ethylene glycol); HLADH, horse liver alcohol dehydrogenase (EC 1.1.1.1); BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; rms, root mean square; EtO^- , ethanolate; iPrO^- , isopropanolate; IPA, isopropanol.

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Fig. 1. Structure of polyethylene glycol bound NAD^+ . R = ribose, P = phosphate, PEG = polyethylene glycol = $(\text{CH}_2\text{-CH}_2\text{-O})_n$.

Several groups have performed modelling studies on this enzyme system, trying to obtain more details about the catalytic and structural features of HLADH [14–16].

In the past we have used a molecular mechanics approach to study in detail the interactions of the coenzyme NAD^+ and a number of analogues in the active site of HLADH, in order to understand the essential factors involved in the productive binding between coenzyme and apo-enzyme [17–20]. In this paper we present the results of detailed kinetic studies on HLADH with PEG- NAD^+ as coenzyme, and an extension of our modelling studies including molecular mechanics (MM) and dynamics (MD), to rationalize the kinetic data obtained.

2. Materials and methods

2.1. Materials

HLADH, NMN^+ and $\beta\text{-NAD}^+$ were purchased from Sigma. PEG- NADH ($M_r = 20\,000$) was supplied by Prof. Dr. C. Wandrey and Dr. U. Kragl from the Institute of Biotechnology in Jülich, prepared according to Ref. [21]. The coenzyme NAD^+ was lyophilised from distilled water. HLADH from Sigma (specific activity 1–2 U/mg) was used because its ethanol content, less than 0.15%, proved to be low enough not to disturb activity determinations using isopropanol as substrate, making extensive dialysis or gel filtration of the enzyme [22] superfluous. The PEG- NADH was oxidized to PEG- NAD^+ as described by Bückmann et al. [21]. The PEG- NAD^+ was purified using a bacterial filter, with a pore size of 0.2 μm . The coenzyme concentrations were determined spectrophotometrically using the following absorption coefficients: $18\,000\text{ M}^{-1}\text{ cm}^{-1}$ at $\lambda_{\text{max}} = 260\text{ nm}$ for NAD^+ , $21\,600\text{ M}^{-1}\text{ cm}^{-1}$ at $\lambda_{\text{max}} = 267.5\text{ nm}$ for PEG- NAD^+ and $6220\text{ M}^{-1}\text{ cm}^{-1}$ at 340 nm in case of the reduced forms. Sodium dithionite was purchased from Janssen Pharmaceutical (purity $\approx 80\%$; $\epsilon = 7000\text{ M}^{-1}\text{ cm}^{-1}$), potassium cyanide from Merck and Koshland reagent from Aldrich (purity 98%; $\epsilon = 18\,000\text{ M}^{-1}\text{ cm}^{-1}$ at $\lambda = 410\text{ nm}$ and pH 10).

2.2. Kinetic measurements

All kinetic experiments were carried out at $25^\circ\text{C} \pm 0.1^\circ\text{C}$ in 47 mM phosphate buffer, 0.25 mM EDTA, $\Gamma = 0.1$.

The pH was adjusted to 8.0 with KOH. A Perkin-Elmer UV/Vis spectrophotometer Lambda 3B was used. Steady-state kinetics were carried out with coenzyme saturation (0.55 mM NAD^+ or 0.59 mM PEG- NAD^+) and a substrate concentration range from 1–10 mM in the case of ethanol and 10–100 mM in the case of isopropanol (see Appendix A for the equation used to determine the initial rates); the NAD^+ and PEG- NAD^+ concentrations were varied between 5–55 μM at a constant ethanol concentration of 10 mM. The change of absorption at 340 nm was measured. The enzyme concentrations ranged from 0.03–0.52 μM during the steady-state measurements. BSA was added to the enzyme stock solution to stabilize the HLADH. The interference of poly(ethylene glycol) in the steady-state kinetics was checked using 1 mM PEG concentration (2%, w/v), 10 mM ethanol and NAD^+ or PEG- NAD^+ (varied between 5–500 μM) as coenzyme.

2.3. Formation of adducts

The rates of formation of sulphinate adducts were determined at 20.1°C in a Hi-Tech Scientific stopped-flow spectrophotometer SF-51. Equal amounts of an anaerobic 0.20 mM sodium dithionite solution in 50 mM Tris-HCl buffer (pH 7.8) and an anaerobic 1.32 mM coenzyme solution in phosphate buffer (pH 8.2) were mixed, under an argon flush. The total volume was 150 μl . The formation of the sulphinate adduct was followed spectrophotometrically by measuring the change in absorption at 353 nm ($\epsilon_{\text{adduct}} = 3200\text{ M}^{-1}\text{ cm}^{-1}$). All the solutions used were made anaerobic by repeatedly flushing with purified argon [23].

The formation of the cyanide adduct was carried out at 25°C in a 0.01 M KCN solution. The coenzyme concentration was 0.17 mM in a total volume of 2.5 ml. The reactions were followed spectrophotometrically by measuring the increase in absorption at 325 nm ($\epsilon_{\text{adduct}} = 6300\text{ M}^{-1}\text{ cm}^{-1}$) [24]. A Perkin-Elmer UV/Vis spectrophotometer Lambda 3B was used.

2.4. Molecular mechanics and dynamics calculations

Energy calculations, total energy-minimizations and molecular dynamics simulations were performed with the molecular mechanical simulation package AMBER 4.0 [25] on an Power Challenge SGI computer. The potential energy function used by AMBER is of the form:

$$V = \sum_{\text{bonds}} k_r (r - r_e)^2 + \sum_{\text{angles}} k_\theta (\theta - \theta_e)^2 + \sum_{\text{dihedrals}} \frac{V_n}{2} (1 + \cos[n\phi - \gamma]) + \sum_{i < j} \left(\frac{A}{r_{ij}^{12}} - \frac{B}{r_{ij}^6} + \frac{q_i q_j}{r_{ij} \epsilon} \right) + \sum_{\text{H bonds}} \left(\frac{C}{r_{ij}^{12}} - \frac{D}{r_{ij}^{10}} \right)$$

The crystallographic structure of the ternary complex of HLADH/NADH/DMSO [13] was used as a starting structure and obtained from the Protein Data Bank (PDB reference number 6ADH). The cysteine residues 46, 103, 111 and 174 were defined as negatively charged cysteines, containing thiolate groups. All histidine residues were taken as HID except the His-51, which was taken HIP (diprotonated histidine). Ethanolate or isopropanolate was substituted at the position of DMSO. Nine water molecules were added manually, according to their subsequent presence in both the binary complex of HLADH (PDB file 5ADH) [12] and the X-ray structure of HLADH with NAD⁺ and pentafluorobenzyl alcohol [6]. Then water was added in a range of 2.9 nm around the catalytic zinc ion, applying a minimum distance of 0.26 nm between the oxygen atom of a water molecule and the heavy atoms of the enzyme complex, resulting in 1368 water residues in the shell around the enzyme. All water molecules were treated as TIP3P residues [26]. Since the enzyme is too large to be minimized completely during the calculations, the belly option was used so that only the amino acid residues within a radius of 1.5 nm around NAD⁺, the alcoholate, the catalytic zinc atom, NAD⁺ and all water molecules were allowed to move freely (6825 atoms in the belly); but first the water molecules and the substrate were optimized, keeping the rest fixed.

The three-dimensional starting structure of PEG-NAD⁺ was constructed from the X-ray NAD⁺ geometry to which the PEG-residues were linked using the program QUANTA/CHARMM 3.3.2 [27]. Only 10 PEG residues plus the spacer group (see Fig. 1) were coupled to the exocyclic adenine-NH₂ group. After the MD heating simulation the

PEG-tail is positioned for the main part of the time on the surface of the water shell.

All energy minimizations (MM-calculations, using 750 steps of steepest descent algorithm, followed by the conjugate gradient algorithm) were performed until the rms gradient value of the energy was less than 0.1 kcal Å⁻¹, using a dielectric constant ϵ of 1 and treating all CH, CH₂ and CH₃ groups of the amino acids as united atoms. The non-bonded pair list had a cutoff of 12 Å and was updated every 25 time steps. The water cap was restrained at the 29 Å boundary by a harmonic potential with a force constant of 0.5 kcal Å⁻¹ (positional constraints). Atomic charges of the nicotinamide moiety, the negatively charged cysteines, iPrO⁻ and of EtO⁻ were derived from 6-31G^{*}/3-21G ab initio calculations (Appendix A Section A.2). The zinc ions carried a charge of 1.45, according to the calculations by Tapia et al. [28]. Most harmonic force constants were obtained directly from the literature or extrapolated (see below, parametrization and Table 1) [29,30].

The molecular dynamics (MD) simulations up to 90 ps were carried out using a time step of 2.0 fs and the SHAKE procedure to constrain all the bond lengths. The following protocol was used: a heating phase of 5 ps to a final temperature of 300 K followed by 35 ps of equilibration and finally an observation period of 50 ps (250 conformations for analysis). Analysis of atomic trajectories was achieved with the CARNAL program [31].

2.5. Parametrization

In order to maintain the tetracoordinated structure of the zinc ion, an explicit Zn–O bond and explicit angles of

Table 1
Newly added AMBER force field parameters

Non-bonded parameters for zinc			
	Zn	$\epsilon = 0.10 \text{ kcal} \cdot \text{mol}^{-1}$	$r^* = 1.40 \text{ Å}$
<i>Angle parameters for NAD⁺</i>			
	CA–C–O	$\theta = 119.8^\circ$	$k_\theta = 80 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
	CA–C–N	$\theta = 115.7^\circ$	$k_\theta = 70 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
	CA–CA–N [*]	$\theta = 119.5^\circ$	$k_\theta = 70 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
	CA–N ^{''} –CA	$\theta = 121.2^\circ$	$k_\theta = 70 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
	CA–N ^{''} –CT	$\theta = 120.6^\circ$	$k_\theta = 70 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
<i>Torsion parameters for NAD⁺</i>			
	CA–CA–C–O	$\gamma = 180^\circ$	$V_2/2 = 0.45 \text{ kcal} \cdot \text{mol}^{-1}$
		$\gamma = 0^\circ$	$V_1/2 = 0.02 \text{ kcal} \cdot \text{mol}^{-1}$
	C#–CA–C–O ^a	$\gamma = 180^\circ$	$V_2/2 = 0.45 \text{ kcal} \cdot \text{mol}^{-1}$
		$\gamma = 180^\circ$	$V_1/2 = 0.02 \text{ kcal} \cdot \text{mol}^{-1}$
	CA–CA–C–N	$\gamma = 180^\circ$	$V_2/2 = 0.45 \text{ kcal} \cdot \text{mol}^{-1}$
		$\gamma = 180^\circ$	$V_1/2 = 0.02 \text{ kcal} \cdot \text{mol}^{-1}$
	C#–CA–C–N ^a	$\gamma = 180^\circ$	$V_2/2 = 0.45 \text{ kcal} \cdot \text{mol}^{-1}$
		$\gamma = 0^\circ$	$V_1/2 = 0.02 \text{ kcal} \cdot \text{mol}^{-1}$
<i>Restraints to maintain tetracoordination around catalytic zinc</i>			
Bond	Zn–O	$r = 2.14 \text{ Å}$	$k_r = 100 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{Å}^{-2}$
Angle	S–Zn–S	$\theta = 132.6^\circ$	$k_\theta = 50 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
Angle	N–Zn–S	$\theta = 107.8^\circ$	$k_\theta = 50 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
Angle	N–Zn–O	$\theta = 95.0^\circ$	$k_\theta = 50 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$

^a C# is the same atom type as CA and only used to describe the torsion of the carboxamide side chain.

N–Zn–S, S–Zn–S and N–Zn–O were inserted between the zinc ion and its ligands (cf. Table 1). The k_r of the Zn–O bond is perhaps a little too low compared with the suggested value in such cases by Ref. [32]. Although they suggest a $r_{eq} = 1.80$ Å, we believe that $r_{eq} = 2.14$ Å is justified, since the observed Zn–O distance in several complexes of HLADH with water or an alcohol are all around this value of 2.14 Å. The parameters describing the dihedral barrier for the carboxamide orientation of the side chain of the nicotinamide moiety with 6-31G* atom charges, were estimated as follows: $V_1/2 = 0.08$ kJ mol⁻¹ (0.02 kcal mol⁻¹) and $V_2/2 = 1.88$ kJ mol⁻¹ (0.45 kcal mol⁻¹) [20]. This resulted in a rotational barrier of 12.6 kJ mol⁻¹ around the C3–C7 bond of the nicotinamide moiety of the free coenzyme.

3. Results and discussion

3.1. Kinetic results

The kinetic behaviour of HLADH with both ethanol and isopropanol can be described by the conventional treatment of those cases in which the enzyme, coenzyme and substrate combine into a ternary complex [33–35], the reaction taking place in this complex. If NAD⁺ is used as coenzyme and ethanol as substrate, the dissociation of the NADH from the enzyme is the rate limiting step, whereas in the case of the poor substrate isopropanol the rate of hydride-transfer is limiting as shown by pre steady-state measurements [36]. Table 2 summarizes the steady-state kinetic data obtained for NAD⁺ and PEG-NAD⁺ ($M_r = 20000$ of the PEG-tail) with ethanol or isopropanol as substrate. The same mechanism as for NAD⁺ is assumed to occur for PEG-NAD⁺. We found that HLADH exhibits substantially lower activity if PEG-NAD⁺ is used instead of the native NAD⁺. The turnover number appears to be three times lower for PEG-NAD⁺/ethanol compared to NAD⁺/ethanol. With isopropanol k_{cat} even drops by a factor of four. With most dehydrogenases similar results

have been found [2–5,37]. Only in a few cases comparable activities for NAD⁺ and PEG-NAD⁺ have been reported [3,4].

The K_M value for PEG-NAD⁺ is significantly larger than the K_M for NAD⁺, which has also been observed with other dehydrogenases [2,5,39].

Since the PEG-NADH sample may contain up to 5% single substituted product (and thus a small amount of free, terminal OH-groups), the interference of poly(ethylene glycol) in the ordinary enzymatic reaction was investigated. Based on experiments in which 2% w/v PEG ($M_r = 20000$) was tested for its properties as substrate or inhibitor for HLADH, it could be concluded that the compound does not interfere in any way, neither with NAD⁺ nor with PEG-NAD⁺.

In order to explain the kinetic results obtained for HLADH with PEG-NAD⁺ as coenzyme we performed several studies including the determination of the intrinsic reactivity of PEG-NAD⁺, binding studies with the apo-enzyme, shifts in the monomer–dimer equilibrium of HLADH and modelling calculations directed at the coenzyme/apo-enzyme interactions.

3.2. Measurements of intrinsic reactivities

The lower activity found with PEG-NAD⁺ in enzymatic reactions with HLADH and many other dehydrogenases might be attributed to a lower intrinsic reactivity of PEG-NAD⁺. We therefore determined the intrinsic reactivities of NAD⁺ and PEG-NAD⁺ using sodium dithionite and potassium cyanide as reducing agents. These reducing agents form sulphinate and cyanide adducts with the cofactors [23,24]. From the results listed in Table 3 it is clear that PEG-NAD⁺ shows a strongly reduced reactivity with both S₂O₄²⁻ and CN⁻ (31% and 43% of the reactivity of NAD⁺, respectively).

From NMR measurements it is known that free NADH in an aqueous environment can adopt a folded conformation, in which stacking occurs between the nicotinamide and adenine rings [40]. Such intramolecular stacking may

Table 2
Kinetic constants of horse liver ADH using NAD⁺ and PEG-NAD⁺ respectively, as coenzymes

Parameter	Units	NAD ⁺		PEG-NAD ⁺	
k_{cat} (EtOH)	s ⁻¹	1.14 ^a	(100%)	0.36	(32%)
k_{cat} (IPA)	s ⁻¹	0.15	(13%)	0.038	(3%)
K_M (NAD ⁺) ^b	μM	16.3		62.0	
K_M (EtOH)	mM	0.46		1.13	
K_M (IPA)	mM	12.3		16.8	
k_{cat} (EtOH)/ K_M (EtOH)	s ⁻¹ mM ⁻¹	2.48	(100%)	0.32	(13%)
k_{cat} (IPA)/ K_M (IPA)	s ⁻¹ mM ⁻¹	0.012	(4.8%)	0.0023	(0.9%)

Initial velocities with varying concentrations of coenzymes or substrates were determined at 25°C, measuring the increase in absorbance at 340 nm. Standard errors for the estimated k_{cat} and K_M values from a fit with HYPER [38] were less than 7% and 20%, respectively of the reported values.

^a This corresponds with a specific activity of the enzyme preparation of 1.6 U/mg. A preparation of 6 U/mg [39] would accordingly have produced k_{cat} (EtOH) of 4.3 s⁻¹.

^b Determined with an ethanol concentration of 10 mM.

Table 3

Determination of the intrinsic reactivities for various coenzymes. The formation of the sulfinate and cyanide adducts were followed spectrophotometrically at 353 nm and 325 nm respectively

Coenzyme	Relative $k(\text{SO}_2^-)$, %	Relative $k(\text{CN}^-)$, %
NAD ⁺	100 ^a	100 ^a
PEG-NAD ⁺	31	43
NMN ⁺	43	59

^a The absolute rates at 0.1 mM dithionite and 10 mM KCN were 0.02 s⁻¹ and 0.0012 Abs·units·s⁻¹, respectively.

very well enhance the reactivity of NAD⁺, as is nicely illustrated by the results obtained for the nicotinamide mononucleotide (NMN⁺), lacking the adenoside part of NAD⁺ (see Table 3). It is conceivable that the poly(ethylene glycol) tail attached to the adenine of PEG-NAD⁺ prevents the stacking phenomenon in PEG-NAD⁺, resulting in the lower intrinsic reactivity observed in solutions in the absence of enzyme¹. It can therefore be argued that the reactivities of NAD⁺ and PEG-NAD⁺, once bound in the active site of the apo-enzyme, do not differ that much; in contrast with the conformational behaviour of free NAD⁺, the coenzyme binds in an extended conformation to HLADH as can be seen in the X-ray structure [13,41].

3.3. Binding studies

The HLADH dimer consists of two subunits, both of which contain one coenzyme binding site and one substrate binding site. Normally, two NAD⁺ residues bind to the HLADH dimer in the coenzyme binding sites, plus two alcohol molecules in the substrate binding pockets. However, either by interactions of two PEG-chains, or after the binding of one PEG-NAD⁺ molecule to a HLADH dimer, steric hindrance from the poly(ethylene glycol) tail, dangling outside the enzyme, with the second site might occur. From experimental data and a mathematical description concerning PEG-properties by Kambe et al. [42,43], it was calculated that the hydrodynamic radius R_h of poly(ethylene glycol) with a M_r of 20 000 is 78 Å. As the linear distance between the entrances of the coenzyme pockets in the dimer is 38 Å, binding of a second PEG-NAD⁺ might be prevented by steric hindrance exerted by the tail of the first bound PEG-NAD⁺. Therefore, the number of coenzyme binding sites available in HLADH after binding of one PEG-NAD⁺ molecule were checked.

¹ Unfortunately we were unable to carry out ¹H-NMR experiments on PEG-NAD⁺ or PEG-NADH. Due to the limited solubility of the polymer bound NAD⁺, the H4 signals could not be measured and no further information about the occurrence of the folded conformation of PEG-NAD⁺ could be obtained.

There are three possibilities altogether after the binding of the first PEG-NAD⁺:

- (1) A second PEG-NAD⁺ is able to bind.
- (2) Only NAD⁺ is able to bind in the other coenzyme binding site, but not a second PEG-NAD⁺.
- (3) The second coenzyme binding site can neither bind PEG-NAD⁺ nor NAD⁺.

Distinction could be made between options 1/3 and 2 by measuring the competition between NAD⁺ and PEG-NAD⁺ (See Appendix A for theoretical background). This competition was measured by determining the activity at constant saturating PEG-NAD⁺ concentration and various subsaturating NAD⁺ concentrations. Fig. 2 shows the theoretical relative rates of coenzyme conversion for the options 1/3 and 2. If option 2 is operative and all dimers contain only one PEG-NAD⁺ at saturating concentration, each dimer still has one coenzyme site available for binding NAD⁺. Adding NAD⁺ should therefore cause a strong increase in rate, the more so as NAD⁺ is more active than PEG-NAD⁺. If on the other hand options 1 or 3 are operative, the increase in rate will be limited, being due only to a partial, competitive replacement of PEG-NAD⁺ by more reactive NAD⁺.

Actually, the experimental results, in which both NAD⁺ and PEG-NAD⁺ are exposed simultaneously to HLADH (see Fig. 2), are in perfect agreement with options 1 and 3, excluding option 2.

Even if the polymer chain is confined within the volume of its hydrodynamic radius, (vide supra), its concentration can be calculated to be only 1.8% w/v, i.e., this is the highest possible concentration of the polymer of the tail of PEG-NAD⁺ bound at the first site that could occur at the second binding site, low enough not to prevent passage of ordinary NAD⁺. As already mentioned in the section dealing with the kinetic results, 2% w/v PEG in the bulk solution indeed does not affect the reaction rate. Therefore option 3 does not occur either, retaining option 1.

Fig. 2. Competition experiments between NAD⁺ and PEG-NAD⁺; calculated (lines, calculated using the equations in Appendix A) and experimental relative rates (■), using a constant, high concentration of PEG-NAD⁺ (0.51 mM).

The remote possibility might be considered that, despite of HLADH being no allosteric enzyme, the nonoptimal binding of the NAD part of PEG-NAD⁺ is transmitted to the other site, causing a similar lower affinity for NAD there. If such were the case, the observed rates would have been intermediate between the two curves in Fig. 2. This possibility can therefore be excluded too.

In summary, the conclusion can be drawn that the poly(ethylene glycol) tail of PEG-NAD⁺ does not interfere with the coenzyme binding to both sites of HLADH.

3.4. Check of the HLADH monomer–dimer equilibrium

It is known from immobilization experiments that HLADH is active only as a dimer [44]. The low activity with PEG-NAD⁺ might therefore be caused by an unfavourable shift in the monomer–dimer equilibrium, producing a partial dissociation of the dimer under the assay conditions. If such an equilibrium disturbance occurs, a decrease of the enzyme concentration at a constant PEG-NAD⁺ concentration would result in a larger amount of inactive HLADH monomer, implying a non-linear enzyme activity vs. enzyme concentration relationship. This possibility was checked by measuring the activity as a function of enzyme concentration in the relevant range of 0.03–0.15 μ M, using ethanol as substrate. Since both coenzymes (PEG-NAD⁺ and NAD⁺) proved to exhibit a linear relationship (results not shown), an equilibrium shift in the presence of PEG-NAD⁺ in the enzyme concentration range concerned, can be ruled out.

All factors investigated thus far are unable to explain the three to four times lower activity of PEG-NAD⁺. The change in k_{cat} (ethanol) suggests that the interaction between enzyme/coenzyme has changed. We therefore examined the possibility that the PEG-tail affects the interactions of the NAD⁺ with the enzyme pocket by carrying out modelling calculations.

3.5. Modelling calculations

In the past, a molecular mechanics approach has been used to study in detail the interactions of the coenzyme NAD⁺ and a number of analogues in the active site of HLADH, in order to understand the essential factors involved in the productive binding between coenzyme and apo-enzyme [17–19,45]. It seemed worthwhile, however, to reconsider their results, utilizing recent X-ray data that show up more water molecules. In the following paragraph we present the results of improved modelling studies, including molecular mechanics (MM) and molecular dynamics (MD), to rationalize the kinetic data obtained.

Several X-ray crystallographic structures of binary and ternary complexes have been determined of HLADH [6–13] which can be used as starting structures for MD-studies aiming at the elucidation of coenzyme/apo-enzyme interactions. The simulations with NADH, NAD⁺ and PEG-

Fig. 3. The total potential (in kcal mol⁻¹) energy versus time (in ps). On the left for the NAD⁺/RO⁻ complexes; on the right for the PEG-NAD⁺/RO⁻ complexes.

NAD⁺ positioned in the ternary complexes of HLADH/DMSO/coenzyme, HLADH/EtO⁻/coenzyme and HLADH/iPrO⁻/coenzyme, using the ternary crystal complex 6ADH [13], will now be discussed.

3.6. Course of the simulations

Variations of the total potential energy of the MD-alkoxide complexes as a function of time are reported in Fig. 3. First the system is being warmed up (300 K) and the energy increases rapidly. Then in the next 45 ps the energy decreases and reaches an equilibrium after ± 60 ps and fluctuates with small deviations around a mean value during the rest of the simulation. Analyses based on the 40–90 ps period respectively the 50–90 ps period, did not lead to different results. Therefore, we used the data obtained from the 40–90 ps analysis period.

3.7. Overall structure

The minimized (PEG-)NAD⁺ structures have a rms deviation of the backbone atoms of the X-ray structures of 0.6 Å for all complexes compared with the X-ray structure. In contrast with the MM geometries, the MD simulations of NAD⁺ and PEG-NAD⁺ complexes (collected from 40–90 ps simulations) show some differences compared with the original X-ray structure, although the overall 3-dimensional structure is maintained (see Fig. 4). It is worth mentioning that between the subunits of the X-ray structure of HLADH/NADH/DMSO rms differences have been observed for the main and side chain atoms of 0.7 and 1.0 Å, respectively [13]. In the recently determined ternary crystal structure with pentafluoro benzylalcohol a rms difference of 0.24 Å between the two subunits has been observed [6].

In Fig. 5 the average rms deviation of the backbone atoms during the 40–90 ps MD simulation are shown

Fig. 4. MD-averaged structures of the $\text{NAD}^+/\text{EtO}^-$ (---) and $\text{PEG-NAD}^+/\text{EtO}^-$ (—) complex and the crystal structure [13] (---). The left side is fixed during the calculations.

(only the atoms included in the belly option are included in the rms deviation). In both cases a plateau has been reached: the plateau for the $\text{NAD}^+/\text{EtO}^-$ complex is higher than the one for $\text{PEG-NAD}^+/\text{EtO}^-$ (1.66 Å versus 1.28 Å). Thus the MD-averaged NAD^+ complex happens to be further away from the original X-ray structure. A similar situation exists for the iPrO^- complexes (see Fig. 5). The oxygen atoms of the nine water molecules explicitly added in the active site show rms deviations from 1.0 to 4.3 Å. Similar rms deviations as reported above, have been communicated by other groups performing MD simulations on proteins [46].

Fig. 5. The rms deviations (in Å) of the backbone atoms for the 250 conformations over 40–90 ps simulation period. On the left the values for the NAD^+ complexes; on the right the ones for the PEG-NAD^+ complexes.

Fig. 6. Stick plot of the MD averaged structures of NAD^+ (---) and PEG-NAD^+ (—) from the iPrO^- complexes.

3.8. Change in the coenzyme position

The primary effect of the PEG-tail is quite evident in Fig. 6, which shows the positions of NAD^+ and PEG-NAD^+ , in case iPrO^- is substrate. It is apparent that the polymer tail pulls the adenine moiety in the outside direction by about 2 Å, dragging along with it the other parts of the coenzyme. A similar results was obtained with ethanol as substrate. This primary effect will of course induce small but significant conformational changes in the coenzyme itself and in the surrounding peptide chain. Some of these will next be treated in detail.

The outer layer of the enzyme complex is not optimally simulated during the calculations, due to the limited number of water molecules around the surface of the enzyme. The polymer tail used in the calculations is relative small (only 10 ethylene glycol residues versus over 400 in the kinetic experiments) and the 'pulling effect' can be expected to be larger when a longer polymer tail is applied. Since a larger water shell around the enzyme surface (and thus around the PEG tail) would diminish this pulling effect, we assume that both errors will sufficiently compensate each other to provide a qualitatively correct picture.

3.9. Changes in the coenzyme conformation

In addition to the shift in position of PEG-NAD^+ shown in Fig. 6, MD results exhibit changes for some of the torsional angles (see Table 4). Most pronounced are those of more than 100° in the case of $\text{PEG-NAD}^+/\text{EtO}^-$ for the torsional angles ζ_N , ζ_A and α_A (Fig. 7), all around

Table 4

Overview of torsion angles ^a (in degrees) for NAD⁺, PEG-NAD⁺ as derived from modelling calculations (MM and MD), and the X-ray structure (NADH/DMSO/HLADH) [13]

Coenzyme	θ	χ_N	γ_N	β_N	α_N	ζ_N	ζ_A	α_A	β_A	γ_A	χ_A
NADH (X-ray)	34	258	39	214	59	207	85	106	147	281	264
<i>MM-results</i>											
NAD ⁺ /iPrO ⁻	13	250	59	181	72	208	79	62	158	296	250
PEG-NAD ⁺ /iPrO ⁻	13	251	59	183	69	209	71	61	146	309	254
NAD ⁺ /EtO ⁻	13	250	58	181	71	206	80	61	157	294	250
PEG-NAD ⁺ /EtO ⁻	13	252	59	184	68	211	69	63	146	309	255
<i>MD-results</i>											
NAD ⁺ /iPrO ⁻	37	242	72	176	76	196	77	43	163	298	251
nPEG-NAD ⁺ /iPrO ⁻	18	251	54	195	68	198	68	66	145	309	254
NAD ⁺ /EtO ⁻	3	230	76	177	62	198	76	43	150	306	252
PEG-NAD ⁺ /EtO ⁻	10	248	39	182	84	318	299	159	122	293	263

The MD time-averaged values are taken from 250 conformations over 40–90 ps.

^a According to IUPAC nomenclature [47].

the phosphate groups. It is remarkable that almost equal differences are observed between the two MD calculations of NADH/DMSO, the one with the limited belly and the one with the free subunit. This similarity suggests that in the present, approximately calculated, structure there are roughly two conformational states, of lower values of ζ_N , ζ_A and α_A , and of higher ones, differing little in their energy minima. Even the removal of a methyl group, i.e., proceeding from PEG-NAD⁺/iPrO⁻ to PEG-NAD⁺/EtO⁻, is sufficient to tip the scales.

Another aspect of possible interest is the decrease of θ from 37° to 18° on adding the PEG-tail to NAD⁺/iPrO⁻ (Table 4). This will be discussed below.

Furthermore, we took a closer look at a few other relevant (torsional) angles. The rotation around the glycosidic bond (torsion χ_N : C2-N1-C1'-O4') indicates the extent of flexibility of the nicotinamide ring in the active site, and its positioning toward the ribose group. The average MD-values (Table 4) for χ_N of 230° (NAD⁺/EtO⁻), 248° (PEG-NAD⁺/EtO⁻), 242° (NAD⁺/iPrO⁻) and 251° (PEG-NAD⁺/iPrO⁻), correspond to an *anti* conformation of the ribose and nicotinamide rings with all complexes simulated, similar to the conformation observed in the X-ray structure [13].

The puckering of the ribose moieties is valued by the magnitude of the torsion angles $\nu_2(N)$ and $\nu_2(A)$ of the nicotinamide and adenine ribose respectively. The MM and MD structures all show $\nu_2(N)$ values similar to the X-ray value of -37° (Table 5). The $\nu_2(A)$ values are

Fig. 7. Nomenclature of the coenzyme torsion angles and the atoms referred to in the text [12,46].

Table 5

Substrate-coenzyme distance and the puckering values of the nicotinamide ring and ribose rings

	X-ray NADH	Minimized structure				MD-averaged structure			
		NAD ⁺ IPRO ⁻	PEG-NAD ⁺ IRPO ⁻	NAD ⁺ ETO ⁻	PEG-NAD ⁺ ETO ⁻	NAD ⁺ IPRO ⁻	PEG-NAD ⁺ IPRO ⁻	NAD ⁺ ETO ⁻	PEG-NAD ⁺ ETO ⁻
C4 (NAD)-C1(RO ⁻) (Å)	4.18 ^a	3.37	3.38	3.42	3.44	3.46	3.57	3.56	3.57
α_C (degrees)	0	-4	-4	-3	-4	4	7	7	10
α_N (degrees)	0	3	3	3	3	-2	1	2	2
$\nu_2(N)$ (degrees)	-37	-38	-38	-38	-38	-32	-34	-33	-36
$\nu_2(A)$ (degrees)	-37	-24	-21	-24	-22	-20	-15	-20	-21

The MD time-averaged values are taken from 250 conformations over 40–90 ps. The X-ray data are taken from the ternary complex with NADH/DMSO/HLADH [13].

^a The C4 (NAD)-S (DMSO) distance is used for the crystal structure.

Table 6

RMS deviations of the MD-averaged structures from the crystal structure and RMS deviations between the two MD-averaged structures of the NAD⁺ and PEG-NAD⁺ complex

	vs. Crystal structure				MD PEG-NAD ⁺ vs. MD NAD ⁺	
	NAD ⁺ iPrO ⁻	PEG-NAD ⁺ iPrO ⁻	NAD ⁺ EtO ⁻	PEG-NAD ⁺ EtO ⁻	EtO ⁻ complex	iPrO ⁻ complex
<i>Backbone atoms</i>	1.45	1.27	1.66	1.28		
<i>Cα residues near coenzyme</i>						
46	0.98	0.91	1.79	0.98	0.85	0.46
47	0.98	0.57	1.92	0.63	1.30	0.41
48	0.65	0.26	1.89	0.40	1.73	0.40
51	0.47	0.20	1.56	0.41	1.55	0.32
67	0.13	0.47	0.11	0.13	0.20	0.52
174	1.06	0.90	1.17	0.84	0.86	0.84
178	1.64	1.06	1.28	1.07	0.40	0.95
202	0.78	0.81	0.98	0.68	1.10	1.46
203	0.83	0.84	0.89	0.68	0.71	1.15
292	0.33	0.33	0.35	0.43	0.63	0.43
317	0.51	0.46	0.69	0.56	0.13	0.30
319	1.91	1.86	1.31	1.55	0.25	0.57
369	0.47	1.56	0.69	1.05	0.52	1.37
<i>Cα residues near enzyme surface</i>						
227	2.46	1.41	2.47	1.02	2.17	1.29
248	3.83	1.36	6.39	2.28	4.42	3.36
297	2.77	3.57	3.16	0.80	2.48	1.69

smaller in the simulated complexes than in the X-ray structure.

The deformation angles α_C and α_N (defined as C2-C6-C3-C4 and C3-C5-C6-N1, respectively) reflect the extent of bending of the nicotinamide ring towards a boat conformation (Table 5). This puckering of the nicotinamide ring places one of the C4-hydrogens of NADH in a pseudoaxial position, which could favour the hydride-transfer rate [48]. In Table 5 the average values for α_C and α_N for NAD⁺ are listed. Plots of these calculated α_C angles versus the

calculated α_N angles are shown in Fig. 8. Only minor differences can be observed between the complexes.

3.10. Changes of the polypeptide chain

Due to some changes in the coenzyme position and geometry, the surrounding peptide chains also exhibits small conformational changes. Deviations of a few amino acids are listed in Table 6. Some C α 's of the coenzyme neighbouring amino acids are displaced by 1–2 Å. Near

Table 7

Hydrogen-bonding distances (in Å) and electrostatic interactions around the phosphate groups of the coenzyme for the time-averaged MD structures and crystal structure

	X-ray	NAD ⁺ iPrO ⁻	PEG-NAD ⁺ iPrO ⁻	NAD ⁺ EtO ⁻	PEG-NAD ⁺ EtO ⁻
O1P _N (NAD)-HN12 (Arg-369)	2.95	1.72		1.82	1.83
O1P _N (NAD)-H (WAT-757)			1.74		
O1P _N (NAD)-H (WAT)			1.69		
O1P _N (NAD)-HN (Val-203)		1.99		2.02	
O2P _N (NAD)-HN (Gly-202)	3.19				
O2P _N (NAD)-HN (Val-203)	3.21				1.83
O2P _N (NAD)-HN (204)		2.14	2.16	1.90	2.14
O1P _A (NAD)-HN11 (Arg-47)	2.91	1.68	1.98	1.70	
O1P _A (NAD)-H (WAT)				1.83	
O1P _A (NAD)-H (WAT)		1.88	2.07	1.83	1.64
O2P _A (NAD)-HN11 (Arg-47)					1.86
O2P _A (NAD)-H (WAT)		2.39	1.65	1.75	
O2P _A (NAD)-H (WAT)		2.11	2.21		1.88
O2P _A (NAD)-HN (Gly-202)		2.32			2.19
O2P _A (NAD)-HNZ1 (Lys-228)	3.29	1.97			

'WAT' denotes a water molecule.

Table 9

Hydrogen-bonding pattern of the carboxamide side chain of the coenzyme with neighbouring amino acids

	X-ray	Minimized structure				MD-averaged structure			
		NAD ⁺	PEG-NAD ⁺	NAD ⁺	PEG-NAD ⁺	NAD ⁺	PEG-NAD ⁺	NAD ⁺	PEG-NAD ⁺
		IPRO ⁻	IPRO ⁻	ETO ⁻	ETO ⁻	IPRO ⁻	IPRO ⁻	ETO ⁻	ETO ⁻
O7 (NAD)-HN (Phe-319)	1.90	1.86	1.86	1.85	1.85	2.02	1.89	1.93	1.98
HNA (NAD)-O (Ala-317)	1.96	1.99	1.97	1.99	1.97	1.90	1.91	1.87	1.93
HNB (NAD)-O (Val-292)	1.73	1.87	1.89	1.87	1.88	2.51	1.89	1.95	1.92

X-ray distances are taken from the crystal conformation [13]; MD distances are averaged for 250 conformations over 40–90 ps. (Distances in Å).

the cleft and therefore the dissociation, explaining the kinetic results with ethanol, where the enzyme-coenzyme dissociation rates are rate-limiting.

In the H-bonding interactions between the carboxamide side chain of the nicotinamide moiety and the enzyme, three amino acids are involved, namely Phe-319, Ala-317 and Val-292. They are shown in Fig. 9 and Table 9. H-bonds are assigned if the donor (D)–acceptor (A) distance is, at least temporary, smaller than 3.5 Å and the D–H–A angle value ranges between 90° and 180°. An H–A bond of ≤ 2.5 Å will be considered as being stable [49]

Fig. 10 shows the fluctuations in the three hydrogen-bonds in these complexes. During the MD simulations all three H-bonds are largely kept intact both in the NAD⁺ and in the PEG-NAD⁺ complexes, with the exception of

the NAD⁺/iPro⁻ Val-292 bond, which is not quite stable (see also the hydride-transfer paragraph).

3.12. The hydride-transfer

Among the factors affecting the rate of hydride-transfer are the distance to be tided over and the out-of-plane orientation of the carboxamide side chain. As far as the first factor is concerned, the data in Table 5 are relevant. The average distances between the C4 of NAD⁺ or PEG-NAD⁺ and the C1 atoms of EtO⁻ and iPro⁻ turn out to be almost equal to the 3.40 Å found by semiempirical calculations (results not explicitly shown here). The angles of approach N1–C4–C1 are also reasonably close; 107° versus 114° for ethanol, and 104° versus 89° for isopropanol for MD and quantum chemical calculations re-

Fig. 10. Hydrogen-bonding distance fluctuations (in Å) between the carboxamide side chain and the HLADH residues Phe-319 (left), Ala-317 (middle) and Val-292 (right).

Fig. 11. The A- and B-side of the nicotinamide ring.

spectively. The PEG-tails do not change the MD values. Apparently all of these data do not provide an explanation for a reduced rate of hydrogen-transfer.

All dehydrogenases show high stereoselectivity towards the hydrogen to be transferred towards C4 of the nicotinamide ring. In A-specific enzymes like HLADH the pro-*R* hydrogen atom H_{4A} is transferred from NADH towards the substrate [50] (see Fig. 11). The H_A and H_B stereospecificity can be explained by the one-sided shielding by the apo-enzyme [51]. The possible relationship between the rate and the direction of hydride transfer and the *syn* orientation of the carbonyl dipole, has been extensively discussed [52–54]. In the X-ray structure of the ternary complex of HLADH/DMSO/NADH the carbonyl group of the side chain is rotated 34° out of the plane of the pyridinium ring [13].

As far as the out-of-plane orientation is concerned, the out-of-plane values θ (O7–C7–C3–C4, Fig. 7) of NAD^+ and PEG- NAD^+ complexes during the simulation period are shown in Fig. 12. For the $NAD^+/iPrO^-$ complex a time-weighted average value of 32° (Table 4) was calculated, i.e., a positioning of the carboxamide side chain on the A-side of the nicotinamide plane, which is in nice agreement with the observed X-ray value of 34° and the 30° calculated by semiempirical studies [55]. On the other hand, the fluctuations in θ in this case (left top of Fig. 12) are larger than those of the other complexes. These fluctua-

tions are accompanied by lengthening of the H-bond with Val-292 (right top of Fig. 10).

Apparently the positioning of the carboxamide side chain is quite flexible.

The $iPrO^-$ complex with the PEG- NAD^+ coenzyme has a smaller average θ than the complex with NAD^+ . Referring to the earlier mentioned relationship between the hydride-transfer rate and the orientation of the carbonyl dipole, this seems to be in agreement with the observed, reduced rate of the hydride-transfer in the case of PEG- NAD^+ [48,52,53].

Not satisfying yet is the average value of θ of only 3° , found for NAD^+/EtO^- . It deviates not only from the X-ray value (which might be explained by the fact that ethanol does not resemble DMSO, present in the crystal structure, as well as isopropanol), but also from the results of the quantum calculations. However, the changes we try to explain are very subtle ones. It may be worthwhile to re-examine this detail by repeating the MD calculation in future with an improved file.

A decrease in rate of hydrogen-transfer when ethanol is the substrate may very well have occurred, owing to the changed interactions between enzyme and coenzyme, particularly in this case. It cannot be detected, however, as the dissociation of the coenzyme is the rate-limiting step.

4. Conclusions

HLADH appears to be three to four times less active with PEG- NAD^+ than native NAD^+ . The following explanations for the kinetic behaviour of PEG- NAD^+ can be ruled out as discussed in the corresponding paragraphs:

- A lower intrinsic reactivity
- Interference of singly substituted poly(ethylene glycol) in the enzymatic reaction
- Steric hindrance exerted by the poly(ethylene glycol) tail
- Destabilization of the HLADH dimer.

The reported MD calculations indicate that the PEG-tail of enzyme-bound PEG- NAD^+ causes shifts in the positioning of the coenzyme and of the surrounding peptide chains, owing to the pulling action of the polymer chain. Such changes are evident with both substrates, ethanolate and isopropanolate. Even previous, less accurate calculations, using DMSO as substrate substitute and including fewer water molecules, exhibited similar changes (not shown). Owing to the small differences in energies, needed for these detected conformational changes, the details of the changes may turn out to be different with future improved calculations, but these are unlikely to affect the main conclusion that there are changes of a sufficient extent to cause a three- to fourfold decrease of activity with PEG- NAD^+ .

Fig. 12. Variation of θ (in degrees) of (PEG-) NAD^+ in HLADH for the 250 conformations during the 40–90 ps sampling phase.

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Appendix A. Competition between two coenzymes C_1 and C_2 at fixed substrate concentration

A.1. System I: normal competition

Conservation equation:

$$e = E + EC_1 + EC_2 \quad (1)$$

e denotes the total concentration of subunits. The dissociation constants K' of the coenzymes at the chosen substrate concentration are:

$$K'_1 = \frac{E \cdot C_1}{EC_1} \text{ and } K'_2 = \frac{E \cdot C_2}{EC_2} \quad (2a, 2b)$$

Insertion into Eq. (1) yields

$$e = E \left(1 + \frac{C_1}{K'_1} + \frac{C_2}{K'_2} \right) = E \cdot A \quad (3)$$

The reaction rate is given by

$$v = k'_1 EC_1 + k'_2 EC_2 \quad (4)$$

in which k'_1 and k'_2 denote the reaction rates with the chosen substrate concentration at infinite coenzyme concentration. Insertion of Eqs. (2) and (3) into Eq. (4) yields Eq. (5) applies to both options 1 and 3. In the latter case only half of e is available to PEG-NAD⁺ but this is then compensated by k' (PEG-NAD⁺) being twice as high (see below, system II).

$$v = \frac{e}{A} \left(k'_1 \frac{C_1}{K'_1} + k'_2 \frac{C_2}{K'_2} \right) \quad (5)$$

A.2. System II: semi competition

If C_1 is the coenzyme that is capable of binding to only one of the two subunits (option 2), it means that only half of the subunits can bind both C_1 and C_2 , according to Eq. (5), and the other half only C_2 . This yields:

$$v = \frac{0.5e}{A} \left(k'_1 \frac{C_1}{K'_1} + k'_2 \frac{C_2}{K'_2} \right) + \frac{0.5ek'_2}{1 + \frac{C_2}{K'_2}} \quad (6)$$

As the experiments were carried out with 10 mM ethanol as substrate, the values $K'_1 = 62.0 \mu\text{M}$ and $K'_2 = 16.3 \mu\text{M}$

could be taken directly from Table 2, whereas k'_1 and k'_2 were calculated from their corresponding values of k_{cat} and K_M , providing the ratio $k'_1/k'_2 = 0.30$. If system II is operative, the observed k_{cat} of PEG-NAD⁺ is due to only one of the two subunits and the actual k_{cat} would be double the one observed. For the calculation of system II the ratio $k'_1/k'_2 = 0.60$ is therefore to be used.

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