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The interactions of artificial coenzymes with alcohol dehydrogenase and other NAD(P)(H) dependent enzymes

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Abstract

The interactions of CL4, a biomimetic analogue of NAD^+ comprising a nicotinamide functionality coupled via a triazine ring to a dibenzenesulphonic acid unit, and of a series of analogues, with HLADH and other dehydrogenases have been compared to those of the natural coenzymes $NAD(P)^+$. CL4, together with one analogue with one of the sulphonic acid groups shifted by one position and another analogue with a single benzenedisulphonic acid unit, have been shown to be functional mimics of NAD^+ in the oxidation of butan-1-ol by horse liver alcohol dehydrogenase (HLADH). A combination of discontinuous HPLC-based assays and continuous fluorescence based assays were used to deduce approximate kinetic constants for this reaction, using the artificial coenzymes, at pH 7.5 and 37°C. HLADH demonstrated a V_{max} with the most active analogue which was 4% of that with NAD^+. The substrate specificity of HLADH using these coenzymes was found to change relative to that using the natural coenzyme. Activity was sought from a range of other dehydrogenases: *Bacillus megaterium* glucose dehydrogenase, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase and sheep liver sorbitol dehydrogenase; all displayed activity using a range of the biomimetic coenzymes.

Keywords: NAD(P)(H); Biomimetic coenzyme; Coenzyme analogue; Dehydrogenases; Coenzyme binding; Horse liver alcohol dehydrogenase

1. Introduction

We have developed a range of coenzyme mimics bearing functional similarity to NAD(P)(H) but composed of more stable, inexpensive and readily synthesised chemical precursors [1–6]. CL4 (Fig. 1) is a simple analogue of the earlier biomimetic coenzymes Blue N-3 and Nap 1. The latter possess anthraquinone and naphthalene groups at the end of the molecule distal from the nicotinamide, while CL4 has only an acetamido group. It is easier to undertake a study of the biochemical properties of CL4 than those of Blue N-3 or Nap 1 because a fluorescence assay may be used to measure the conversion of CL4 to its reduced form. The reduced form of the coenzyme fluoresces at 450 nm when excited at 350 nm [5]. The same fluorescence method can be used for NAD^+,

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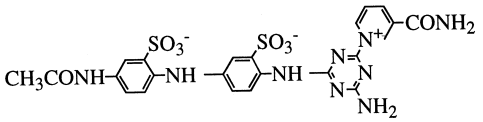
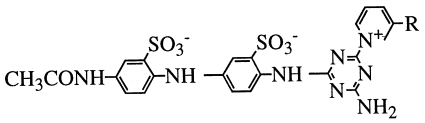


CL4



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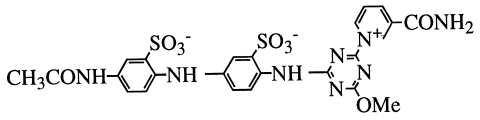

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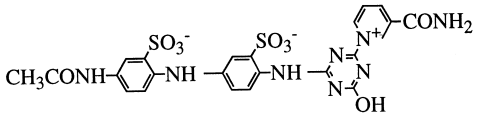

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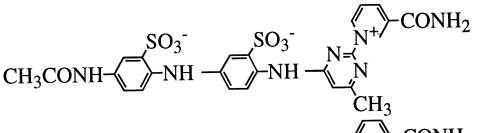

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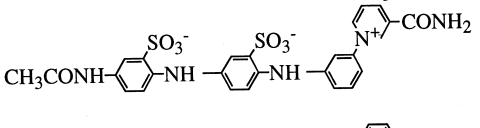

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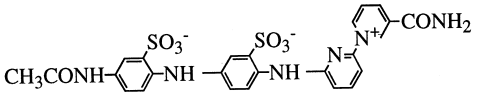

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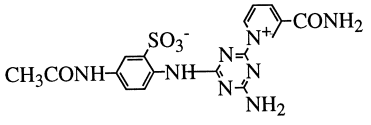

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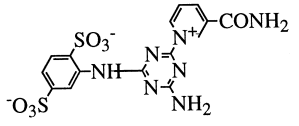

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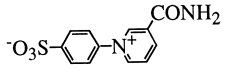

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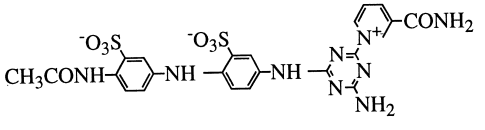

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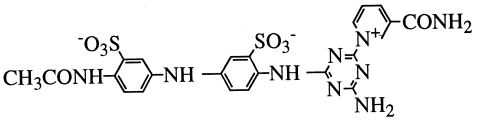

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Fig. 1. Chemical structures of CL4 and of the analogues **1–13**.

providing a direct comparison. The analogues **1–13** were synthesised as close structural analogues of CL4 [6], to investigate the effect of a range of small structural alterations on the interactions of the artificial coenzymes with HLADH and other dehydrogenases. The analogues **10** and **13** were shown to be active in the oxidation of butan-1-ol by HLADH, and to give similar fluorescence changes to CL4 [6].

HLADH is an exceptionally well characterised enzyme [7], and much is known about its interactions with NAD^+. Even subtle changes in the coenzyme structure have been shown dramatically to effect its activity [8,9] although HLADH has in general proven to be more tolerant of such changes than other enzymes. We sought to understand better the differences in the behaviour of HLADH using the various new coenzyme analogues by obtaining approximate kinetic constants for the oxidation of butan-1-ol using CL4, **10** or **11**. The substrate specificity of HLADH using the different coenzyme analogues was also investigated; the broad substrate specificity exhibited by HLADH [10,11] is one reason for the interest in this enzyme as a potential industrial catalyst.

A number of dehydrogenases other than HLADH have previously been tested for activity using Blue N-3 as coenzyme [2], though activity was detected from only two; recombinant β1β1 alcohol dehydrogenase from human liver (hβ1β1ADH) and sorbitol dehydrogenase from sheep liver. Both enzymes are members of the long chain alcohol dehydrogenase family and possess amino acid sequences closely related to that of HLADH [12]. It is possible that for other enzymes Blue N-3 may simply not fit into the coenzyme binding site in the same way as NAD^+; in this case the smaller structure of CL4 may be more likely to demonstrate activity. In the present work, these and other dehydrogenases were tested for activity using CL4, **10** and **13** as coenzyme.

Although only two of the analogues **1–13** (**10** and **13**) were previously found to be active in the oxidation of butan-1-ol by HLADH [6], it is possible that some of the other analogues may display reduced, or zero, coenzymic activity with HLADH, but increased coenzymic activity with other dehydrogenases. The electrochemical properties of the remaining analogues suggest that some are stronger oxidants than CL4, and from a purely thermodynamic viewpoint should function as superior NAD^+ analogues [6]. Chemical reduction of these analogues led to changes in the fluorescence and/or UV–visible absorption properties. Thus, similar changes were sought when these remaining analogues were incubated with a similar range of dehydrogenases as CL4.

The adenine ring of NAD^+ has been shown to make a large contribution to the coenzymic activity with HLADH [13]. Because none of the analogues **1–13** possess a large aromatic group such as anthraquinone (Blue N-3) or naphthalene (Nap 1) to mimic the adenine of NAD^+, they were not expected to demonstrate high coenzymic activity. However, any modifications of the structure of CL4 which lead to improved coenzymic properties over CL4, may be paralleled by similar improvements for the equivalent structural changes to Blue N-3 or Nap 1, which could lead to an artificial coenzyme of real commercial value.

2. Experimental

2.1. Materials

All reagents were analytical grade and were purchased from BDH (Merck), Sigma or Aldrich. Horse liver alcohol dehydrogenase (HLADH) was from Sigma. Enzyme solutions were prepared and concentrations measured as described previously [4]. The artificial coenzymes CL4 and **1–13** were as synthesised previously. Concentrations of coenzyme analogues in solution were calculated using extinction coefficients $\varepsilon_M CL4_{318\ nm} = 24{,}000\ l\ mol^{-1}\ cm^{-1}$, $\varepsilon_M \mathbf{10}_{266\ nm} = 18{,}000\ l\ mol^{-1}\ cm^{-1}$ and $\varepsilon_M \mathbf{13}_{310\ nm} = 26{,}000\ l\ mol^{-1}\ cm^{-1}$. Concentrations of other analogues were calculated on

the basis of weight using theoretical molecular masses.

2.2. Analytical techniques

A Hewlett-Packard gradient HPLC system was used comprising: 1050 series quaternary pump with column oven, 1050 series multiple wavelength detector, 1050 series 21 vial autosampler and a Zorbax 5 μm octadecyl (C18) silane column (250 mm × 4.6 mm) maintained at 40°C with a 20 mm × 2 mm pre-column; data were processed by Hewlett-Packard Chemstation software on a Laser 486/3. The column was equilibrated with methanol–0.1% (w/v) aqueous *N*-cetyltrimethylammonium bromide (70:30, v/v) for several hours before use at a flow rate of 1.5 ml min^{-1}. Injected samples (20 μl) were eluted at 1.2 ml min^{-1} using the following gradient profile: time 0 min, aqueous solvent 30%; 10.0 min, 10%; 10.1 min, 0%; 14.0 min, 0%; 14.1 min, 30%; 22.00 min, 30%. This elution strategy was adapted from that used to separate the oxidised and reduced forms of Blue N-3 [1,2]. Eluted peaks were detected by absorption at 254 or 340 nm.

2.3. Roche Cobas Fara fluorescence assays

Reaction rates were measured using a Roche Cobas Fara Centrifugal Analyser. Although this instrument is not as accurate as a dedicated fluorimeter, it can measure up to 30 reactions simultaneously and uses very small volumes; hence, very high concentrations of enzyme could be used to screen for coenzymic activity.

In a total volume of 300 μl in a well of a Roche Cobas Fara rotor were enzyme (differing concentrations), coenzyme/analogue (200 μM), sodium phosphate (40 mM) buffer pH 7.50, substrate (differing concentrations). Enzyme, coenzyme/analogue and buffer were pre-equilibrated at 37°C. The reaction was initiated by the addition of substrate and the rate of change in fluorescence (excitation 350 nm, emission 450 nm) measured over 10 min (measurements every 30 s). Rates were compared to standards, measured in triplicate. For measurements with $NAD(P)^+$: NAD^+ (200 μM), HLADH (5 nM), sodium phosphate (40 mM) buffer pH 7.50, butan-1-ol (100 mM). For CL4 and **10**: similarly but 200 μM CL4 or **10**, and 4 μM HLADH. For **13**: similarly but 200 μM **13**, and 0.8 μM HLADH.

2.4. Comparison of steady-state kinetic parameters for the oxidation of butan-1-ol by HLADH at 37°C and pH 7.5 using NAD^+ or the artificial coenzymes

2.4.1. Rates with varying concentrations of NAD^+ at 100 mM butan-1-ol

HLADH (5.0 nM), NAD^+ (0–200 μM), sodium phosphate (40 mM) buffer pH 7.50 and butan-1-ol (100 mM) were incubated in 1.0 ml total volume at 37°C. Reaction was initiated by the addition of butan-1-ol and the increase in absorption at 340 nm monitored using a Hewlett-Packard HP8452A diode array spectrophotometer which calculated reaction rates using $\varepsilon_M NADH_{340\,nm} = 6220\ l\ mol^{-1}\ cm^{-1}$. Each assay was performed in triplicate. Data were fitted to the Michaelis–Menten equation using the computer programme GRAFIT [14].

2.4.2. Rates with varying concentrations of artificial coenzyme at 100 mM butan-1-ol

HLADH (4.0 μM), CL4 (0–400 μM), sodium phosphate (40 mM) buffer pH 7.50 and butan-1-ol (100 mM) were incubated in a total volume of 200 μl at 37°C. Reaction was initiated by the addition of butan-1-ol. After exactly 10 min, 50 μl were withdrawn, diluted 1:1 with DMSO to stop the reaction, and analysed by HPLC. The reaction rate was calculated from the amount of reduced CL4 produced in 10 min, determined from the HPLC chromatogram using the ratio $\varepsilon_M(\text{reduced CL4})_{340\ nm}/\varepsilon_M CL4_{340\ nm} = 1.44$. This ratio was determined by allowing an assay to proceed to completion. Each assay was performed in duplicate. Controls with no HLADH were performed. The chromatograms showed only CL4 to be present. Data were

fitted to the Michaelis–Menten equation using the computer programme GRAFIT [14]. Assays were performed similarly for **13**, but with 0.4 μM HLADH. Reaction rates were calculated from the amount of reduced **13** produced in 10 min, determined from the HPLC chromatograms using ε_M(reduced **13**)$_{340\ nm}/\varepsilon_M$**13**$_{340\ nm}$ = 1.69. Assays were performed similarly for **10**, but with 15.8 μM HLADH. A slow background rate due to hydrolysis of **10** was subtracted, and reaction rates were calculated from the amount of reduced **10** produced in 10 min, determined from the HPLC chromatograms using ε_M(reduced **10**)$_{254\ nm}/\varepsilon_M$**10**$_{254\ nm}$ = 0.738.

2.4.3. Rates with varying concentrations of butan-1-ol at 200 μM NAD$^+$/coenzyme analogue

HLADH (10 nM), NAD$^+$ (200 μM), sodium phosphate (40 mM) buffer pH 7.50 and butan-1-ol (0.6 to 100 mM) were incubated in the Roche Cobas Fara fluorescence assay. Assays were performed in duplicate. Data were fitted to the Michaelis–Menten equation using the computer programme GRAFIT [14]. Assays were performed similarly for CL4, **10** and **13**, using 4.0 μM, 4.0 μM and 0.8 μM HLADH, respectively.

2.5. Comparison of the substrate specificity of HLADH using different coenzymes

HLADH (5.0 nM), NAD$^+$ (200 μM), sodium phosphate (40 mM) buffer pH 7.50 and alcohol substrate (50 mM) were incubated in the Roche Cobas Fara fluorescence assay. Assays were performed in duplicate. For CL4, **10** and **13**, 4.0 μM, 4.0 μM and 0.8 μM HLADH were used, respectively. The coenzyme concentration was always 200 μM.

2.6. Comparison of the activity of other dehydrogenases using different coenzymes

Yeast alcohol dehydrogenase (EC 1.1.1.1) (yADH), *Thermoanaerobium brockii* alcohol dehydrogenase (NADP$^+$-dependent) (EC 1.1.1.1) (*Tb*ADH), *Bacillus subtilis* L-alanine dehydrogenase (EC 1.4.1.1) (*Bs*AlaDH), *Bacillus megaterium* glucose dehydrogenase (EC 1.1.1.47) (*Bm*GluDH), *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (*Lm*G6PDH), *Cellulomonas species* glycerol dehydrogenase (EC 1.1.1.29) (*Cs*GlyDH), *Enterobacter aerogenes* glycerol dehydrogenase (EC 1.1.1.29) (*Ea*GlyDH), *Aspergillus niger* glycerol dehydrogenase (NADP$^+$-dependent) (EC 1.1.1.29) (*An*GlyDH), porcine heart *threo*-D$_s$-isocitrate dehydrogenase (NADP$^+$-dependent) EC 1.1.1.42) (phIDH), and sheep liver sorbitol dehydrogenase (EC 1.1.1.14) (slSDH) were purchased from Sigma. *Pseudomonas putida M10* morphine dehydrogenase (morphine:NADP$^+$ oxidoreductase) (*Pp*MDH) was a kind gift of Dr. N.C. Bruce, University of Cambridge Institute of Biotechnology. Recombinant human β1β1 alcohol dehydrogenase (hβ1β1ADH), human β2β2 alcohol dehydrogenase (hβ2β2ADH) and human β3β3 alcohol dehydrogenase (hβ3β3ADH) were kind gifts of Prof. W.F. Bosron, Indiana University Medical School, IN, USA.

hβ1β1ADH in 50% (v/v) glycerol was dialysed (16 h) into sodium phosphate (100 mM) buffer pH 7.50 containing dithiothreitol (0.5 mM) and zinc sulphate (1.0 μM) and concentrated in a stirred cell concentrator. The solution was heated to 52°C for 10 min and centrifuged at 13,000 rpm for 5 min in an MSE MicroCentaur to remove denatured protein. The supernatant was removed and its activity was measured under identical conditions as for HLADH [15].

hβ2β2ADH and hβ3β3ADH were treated similarly. For hβ2β2ADH activity was measured at 25°C in sodium pyrophosphate (100 mM) buffer pH 8.50 with NAD$^+$ (2.5 mM) and ethanol (33 mM), and for hβ3β3ADH at 25°C in sodium phosphate (100 mM) buffer pH 7.00 with NAD$^+$ (2.5 mM) and ethanol (66 mM). Enzyme concentrations were calculated using specific activities of 0.24 units mg^{-1},

19 units mg^{-1} and 7.9 units mg^{-1} for hβ1β1ADH, and hβ2β2ADH and hβ3β3ADH, respectively [15,16].

*Bs*AlaDH (EC 1.4.1.1) in 50% (v/v) glycerol (0.46 ml, 1.5 mg protein ml^{-1}) was equilibrated in sodium phosphate (100 mM) buffer pH 7.50 using a Pharmacia PD-10 column, concentrated to 1.2 ml in a Filtron centrifugal concentrator and the enzyme concentration calculated to be 0.162 mg ml^{-1}.

For the remaining enzymes, a known weight was reconstituted in a known volume of sodium phosphate (100 mM) buffer, pH 7.50, and the approximate concentration obtained by calculation.

The Roche Cobas Fara fluorescence assay was used to detect activity from the different enzymes using NAD^+, CL4, **1–5**, **10**, **12** and **13**. For **1–5** and **12**, the same standards were used as for CL4. For **6**, **7**, **8** and **11** the Roche Cobas Fara was set to monitor absorption rather than fluorescence (at 400, 500, 500 and 400 nm, respectively). The concentration of coenzyme was always 200 μM. **9** was omitted from this study owing to its poor solubility.

3. Results and discussion

CL4, **10** and **13** have previously been shown to function coenzymically in the oxidation of butan-1-ol by HLADH. In each case, reduction of the coenzyme analogue leads to increased fluorescence at 450 nm in the presence of an excitation at 350 nm. This provides a convenient method for following enzymic reactions which is more sensitive than UV–visible absorption spectroscopy [5,6]. However, there are problems associated with the fluorescence assay: Since all the coenzyme analogues absorb at 350 nm in their native (oxidised) forms, the amount of incident light is reduced. This inner filter effect restricts the coenzyme concentration to less than 300 μM. The rate of increase of fluorescence for each coenzyme is a complicated function of the amounts of oxidised and reduced coenzyme, the extinction coefficient at 350 nm and the quantum yield. Thus, for each coenzyme, and for each concentration, the actual rate of production of the reduced species must be calculated by comparison with a standard. The standards were chosen to be 200 μM in coenzyme and 100 mM in butan-1-ol. Actual rates were calculated from a spectrophotometric assay for NAD^+ and from HPLC assays for CL4, **10** and **13**.

In order to compare the behaviour of the coenzyme analogues more closely with NAD^+, kinetic constants were determined for the oxidation of butan-1-ol by HLADH at pH 7.5 and 37°C using the various coenzymes. The general initial rate equation describing two-substrate enzyme kinetics is

$$\frac{V}{[\mathrm{E}]} = \frac{V_{\max}[\mathrm{A}][\mathrm{B}]}{K' \cdot K_{\mathrm{mA}} + K_{\mathrm{mA}}[B] + K_{\mathrm{mB}}[\mathrm{A}] + [\mathrm{A}][\mathrm{B}]}$$

where A, B are the two substrates and V = the observed rate; [E] = the concentration of enzyme; $V_{\max}$ = the maximum theoretical rate; K_{mA} = the Michaelis constant for A; K_{mB} = the Michaelis constant for B; K = the two substrate constant.

The equation should, strictly, be solved by varying A and B simultaneously to produce a matrix of data. However, if A or B is saturating, the kinetics approximate to the Michaelis–Menten case for B or A, respectively. If K' is small, the Michaelis constant measured for A at saturating B will approximate to the true K_{mA} and vice versa. Thus, K_{m} for NAD^+ was determined by measuring the absolute rates at 100 mM butan-1-ol and varying NAD^+ concentrations using the spectrophotometer (Fig. 2a). A total of 200 μM NAD^+ was determined to be essentially saturating. Rates were measured at 200 μM NAD^+ and varying butan-1-ol concentrations by fluorimetry giving K_{m} for butan-1-ol

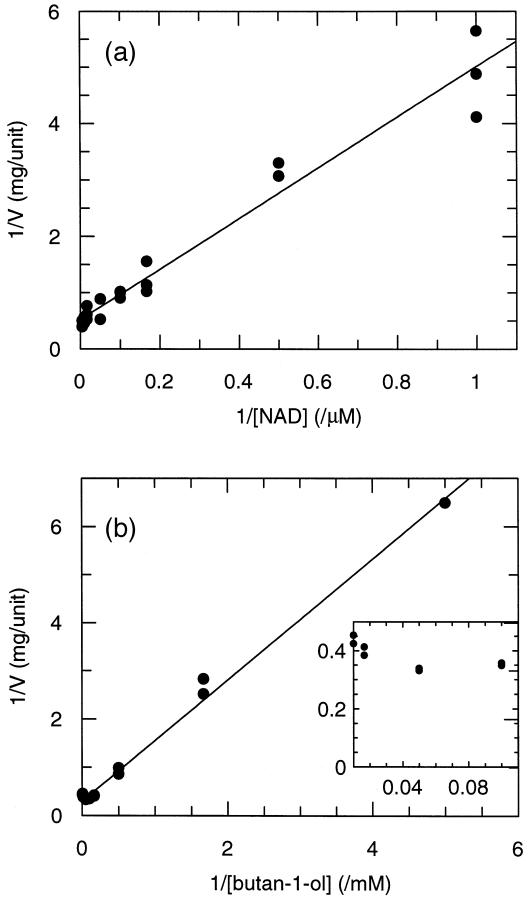

Fig. 2. Dependence of the rate of butan-1-ol oxidation by HLADH on the concentration of NAD^+ and of butan-1-ol, measured at pH 7.5 and 37°C. (a) Lineweaver–Burke plot of $1/V$ against $1/[NAD^+]$, at constant butan-1-ol (100 mM). (b) Lineweaver–Burke plot of $1/V$ against $1/$[butan-1-ol], at constant NAD^+ (200 μM).

and V_{max} when NAD^+ is used as coenzyme (Fig. 2b). The calculation of K_m for butan-1-ol is complicated by the decrease in rate with increasing butan-1-ol concentration above 20 mM. This substrate inhibition has been attributed to the formation of an abortive HLADH · NADH · alcohol complex [10].

Since K_m for NAD^+ was determined at 100 mM NAD^+, these results are approximations to the true values in the two substrate kinetics equation.

K_ms for CL4, **10** and **13** were determined by measuring the absolute rates at 100 mM butan-1-ol and varying coenzyme analogue concentrations using an HPLC assay. When the reaction was stopped, reduction of the coenzyme analogue was in each case shown to be less than 20%. Thus, the calculated rates were approximate initial rates. In each case the K_m for the coenzyme analogue was found to be greater than 200 μM (Fig. 3a). However, since the fluorescence assay was limited to an upper coenzyme concentration of about 300 μM, rates were measured for varying butan-1-ol concentrations using 200 μM coenzyme analogue, giv-

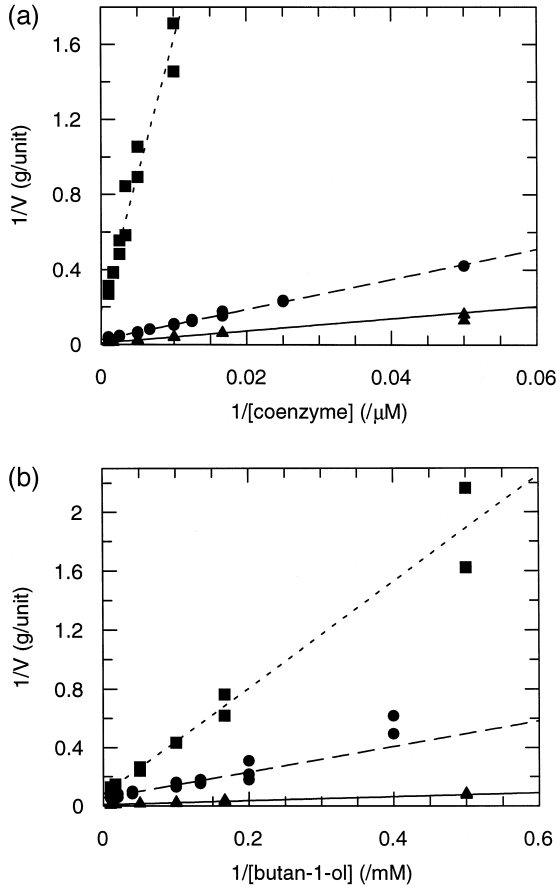

Fig. 3. Dependence of the rate of butan-1-ol oxidation by HLADH on the concentration of artificial coenzymes CL4 (circles, dashed curve), **10** (squares, dotted curve) and **13** (triangles, continuous curve), and of butan-1-ol, measured at pH 7.5 and 37°C. (a) Lineweaver–Burke plot of $1/V$ against $1/$[coenzyme] at constant butan-1-ol (100 mM). (b) Lineweaver–Burke plot of $1/V$ against $1/$[butan-1-ol], at constant coenzyme (200 μM).

ing an approximate K_m for butan-1-ol (Fig. 3b). Under the experimental conditions the rates of increase of fluorescence were essentially linear. For the artificial coenzymes no substrate inhibition was observed at high butan-1-ol concentrations (Fig. 3b). Approximate V_{max}s were calculated by extrapolating the V_{max}s calculated at 200 μM coenzyme to saturating concentrations of the coenzyme analogues.

The results obtained for the approximate kinetic parameters are shown in Table 1. In comparison to NAD^+, each of the artificial coenzymes exhibits higher values of K_m for both coenzyme and butan-1-ol, and lower values of V_{max}, as has previously been observed for other NAD^+ analogues [13]. The oxidation of ethanol by HLADH using NAD^+ as coenzyme has been shown to obey the compulsory order mechanism proposed by Theorell and Chance [17] in the pH range 5.3–9.9 [18]. According to this mechanism, the rate determining step at saturating NAD^+ and ethanol (i.e., V_{max}) is the dissociation of NADH. Thus, if HLADH oxidises butan-1-ol according to the Theorell–Chance mechanism, the reduced forms of CL4, **10** and **13** must dissociate much more slowly than NADH. Alternatively, a different mechanism from the Theorell–Chance may be operative, or another step may be rate-determining, for instance the transfer of hydrogen from butan-1-ol to the coenzyme. This step would be expected to be slower if the nicotinamide were not optimally positioned. In this case, the differing position of the sulphonate group on **13** relative to CL4 may serve to position the nicotinamide group better. Only a more detailed kinetic analysis would enable the actual mechanism operating to be clearly identified.

Table 1
Approximate steady state kinetic parameters for NAD^+ and the coenzyme analogues CL4, **10** and **13** in the oxidation of butan-1-ol by HLADH at pH 7.5 and 37°C

	NAD^+	CL4	**10**	**13**
$K_{m(Coenzyme)}$ (μM)	11.1	239	1210	365
$K_{m(Butan-1-ol)}$ (mM)	4.11	15	37	24
V_{max} (units mg^{-1})	3.80	0.0368	0.0110	0.145
k_{cat} (s^{-1})	2.53	0.0245	0.0073	0.0967
$k_{cat}/K_{m(Coenzyme)}$ (M^{-1} s^{-1})	227,900	103	6.05	265
$k_{cat}/K_{m(Butan-1-ol)}$ (M^{-1} s^{-1})	616	1.63	0.198	4.03

Table 2
Comparison of the rates of oxidation of various alcohols by HLADH using the natural coenzyme NAD^+ or the artificial coenzymes CL4, **10** or **13**, measured by fluorescence at pH 7.5 and 37°C

	NAD^+	CL4	**10**	**13**
Primary alcohols				
Ethanol	6440	0	0.03	2.2
Propan-1-ol	5280	2.5	0.31	13.3
Butan-1-ol	2650	14.0	0.94	31.2
Pentan-1-ol	2400	9.0	0.63	19.9
Hexan-1-ol	3940	4.3	0.38	11.6
Heptan-1-ol	3750	5.1	0.55	9.7
Octan-1-ol	1830	2.9	0.22	7.5
Secondary alcohols				
Propan-2-ol	650	0	0	1.1
Butan-2-ol	1230	0	0.08	5.5
Cyclohexanol	8940	0	0.15	1.1
Branched alcohols				
2-Methylpropan-1-ol	6130	2.3	0.20	6.4
2-Methylbutan-1-ol	5280	4.1	0.28	12.4
Branched secondary alcohols				
3-Methylbutan-2-ol	1980	2.5	0.55	7.5
Aromatic Alcohols				
Benzyl alcohol	480	0.6	0.22	3.6
Diols				
Ethylene glycol	1400	0	0	0
Unsaturated alcohols				
2-Propen-1-ol	4710	2.6	0.64	3.1

Rates are expressed in units/g (μmol min^{-1} g^{-1}), conditions as in text.

Butan-1-ol has been found to be the best substrate for HLADH when Blue N-3 is used as coenzyme [2]. Measurements of the rates of oxidation of various alcohols (50 mM) by HLADH using NAD^+, CL4, **10** or **13** (all 200 μM) as coenzyme were made at pH 7.5 and 37°C (Table 2). The observed substrate specificities for all the artificial coenzymes are very similar, with butan-1-ol being the most favoured substrate, although **10** and **13**, unlike CL4, ap-

pear to be coenzymically active in the oxidation of secondary alcohols. With both **10** and **13**, activity is also detected using ethanol as substrate, although it is very low compared to the activity with butan-1-ol. This is also true for Blue N-3 and confirms that for the binary complex of HLADH with the artificial coenzymes, the structure of the substrate binding site must differ from that induced by the binding of NAD^+, such that a longer hydrophobic chain is necessary to hold the alcohol group in a productive orientation for hydrogen transfer. Another possible explanation for the observed change in substrate specificity is that the artificial coenzymes, or protein residues displaced by coenzyme binding, project into the substrate binding site. The NAD^+ analogue 3-benzoylpyridine adenine dinucleotide, which has a bulky phenyl ring attached to the nicotinamide moiety, functions coenzymically with HLADH, but with a similar decrease in the range of alcohols oxidised [19].

Measurements of the rates at which various other dehydrogenases were able to oxidise their substrates using NAD^+, CL4, **10** or **13** as coenzyme were made at pH 7.5 and 37°C (Tables 3 and 4).

hβ1β1ADH bears a high degree (87%) of sequence identity to HLADH [20] and differs by only one residue at the coenzyme binding site, Ser-48, which has been conservatively replaced by threonine in the human enzyme. hβ1β1ADH accepts CL4 and **13** as coenzyme, with similar changes in substrate specificity as observed for

Table 3
Comparison of the activities of various dehydrogenases using the natural coenzyme NAD^+ ($NADP^+$ for *Tb*ADH) or the artificial coenzymes, CL4, **10** or **13**, measured by fluorescence at pH 7.5 and 37°C using 200 μM coenzyme

Enzyme	Substrate	$NAD(P)^+$	CL4	**10**	**13**
hβ1β1ADH[a]	Ethanol	81	0	0	0.73
	Butan-1-ol	60	0.61	0	1.12
	Propan-2-ol	71	0	0	0
	2-Methylpropan-1-ol	72	0.54	0	0.16
hβ2β2ADH[b]	Ethanol	2610	0	0	0
	Butan-1-ol	1750	0	0	0
	Propan-2-ol	0	0	0	0
	2-Methylpropan-1-ol	1840	0	0	0
hβ3β3ADH[c]	Ethanol	109	0.66	0	0
	Butan-1-ol	199	0	0	0
	Propan-2-ol	0	0	0	0
	2-Methylpropan-1-ol	57	1.55	0	0.53
yADH[d]	Ethanol	1140	0	0	0
	Butan-1-ol	1000	0	0	0
	Propan-2-ol	1000	0	0	0
	2-Methylpropan-1-ol	0	0	0	0
*Tb*ADH[e]	Ethanol	2400	0	0.90	0
	Butan-1-ol	0	0	0.63	0
	Propan-2-ol	36,700	0	2.20	0
	2-Methylpropan-1-ol	27,500	0	0	0

Rates are expressed in units/g (μmol min^{-1} g^{-1}), enzyme concentrations as below.
[a]For NAD^+: 8.92 μg ml^{-1}; for CL4, **10** and **13**: 1.78 mg ml^{-1}.
[b]For NAD^+: 0.17 μg ml^{-1}; for CL4, **10** and **13**: 0.17 mg ml^{-1}.
[c]For NAD^+: 1.37 μg ml^{-1}; for CL4, **10** and **13**: 0.82 mg ml^{-1}.
[d]For NAD^+: 0.20 μg ml^{-1}; for CL4, **10** and **13**: 0.40 mg ml^{-1}.
[e]For NAD^+: 88 ng ml^{-1}; for CL4, **10** and **13**: 176 μg ml^{-1}.

Table 4
Comparison of the activities of various dehydrogenases using the natural coenzymes NAD^+ or $NADP^+$ or the artificial coenzymes CL4, **10** or **13**, measured by fluorescence at pH 7.5 and 37°C with 200 μM coenzyme

Enzyme	$NAD(P)^+$	CL4	**10**	**13**
*Bs*AlaDH[a]	1770 (NAD^+)	0	0	0
*Bm*GluDH[b]	1950 (NAD^+)	0	0.93	8.15
*Lm*G6PDH[c]	132,7000 (NAD^+)	167	0.51	88.8
*Cs*GlyDH[d]	16,400 (NAD^+)	0	0	0
*Ea*GlyDH[e]	602 (NAD^+)	0	0	0
*An*GlyDH[f]	761 ($NADP^+$)	0	0	0
phIDH[g]	~1000 ($NADP^+$)	0	0	2.51
*Pp*MDH[h]	7620 ($NADP^+$)	0	0	0
slSDH[i]	465 (NAD^+)	2.55	1.69	5.10

Rates are expressed in units/g (μmol min^{-1} g^{-1}), enzyme concentrations as below.
[a]For NAD^+: 32.4 ng ml^{-1}; for analogues: 64.8 μg ml^{-1}. Substrate: L-alanine (20 mM).
[b]For NAD^+: 3.24 μg ml^{-1}; for analogues: 32.4 μg ml^{-1}. Substrate: glucose (50 mM).
[c]For NAD^+: 2.9 ng ml^{-1}; for analogues: 58 μg ml^{-1}. Substrate: glucose-6-phosphate (5.0 mM).
[d]For NAD^+: 0.20 μg ml^{-1}; for analogues: 0.10 mg ml^{-1}. Substrate: glycerol (100 mM).
[e]For NAD^+: 9.0 μg ml^{-1}; for analogues: 0.18 mg ml^{-1}. Substrate: glycerol (100 mM).
[f]For $NADP^+$: 2.68 μg ml^{-1}; for analogues: 26.8 μg ml^{-1}. Substrate: glycerol (10 mM).
[g]For $NADP^+$: 3.48 μg ml^{-1}; for analogues: 0.348 mg ml^{-1}. Substrate: *threo*-$D_S L_S$-isocitric acid trisodium salt (100 mM).
[h]For $NADP^+$: 0.272 μg ml^{-1}; for analogues: 27.2 μg ml^{-1}. Substrate: codeine (50 mM).
[i]For NAD^+: 80.9 ng ml^{-1}; for analogues: 0.404 mg ml^{-1}. Substrate: sorbitol (100 mM).

HLADH, but not **10**. hβ2β2ADH differs from hβ1β1ADH by only one residue, Arg-47, which becomes histidine in hβ2β2ADH. This residue is important in the binding of coenzyme [21], and its substitution increases the rate of dissociation of coenzyme from the hβ2β2ADH·NADH complex such that hβ2β2ADH exhibits much faster steady-state kinetics for the oxidation of alcohols using NAD^+ as coenzyme [15]. However, no activity was detected with hβ2β2ADH using the artificial coenzymes. hβ3β3ADH differs from hβ1β1ADH only by the substitution of cysteine for Arg-369. hβ3β3ADH accepts CL4 and **13** as coenzyme, with changes in substrate specificity, but not **10**. These results suggest that Arg-47 is a crucial residue for the activity of the artificial coenzymes, and that CL4 and **13** are more tolerant of changes to other residues at the binding site than is **10**.

No activity was detected with yADH or with *Tb*ADH when CL4 or **13** was used as coenzyme. Both enzymes belong to the long chain alcohol/polyol dehydrogenase family. yADH is a tetrameric enzyme related to HLADH (but less closely than the human alcohol dehydrogenases), with subunits showing 25% sequence identity to HLADH [22]. *Tb*ADH is a tetrameric, $NADP^+$-dependent enzyme, with subunits showing 25% sequence identity to HLADH but more closely related to yADH [23]. **10** unexpectedly appears to be active with TbADH. However, the analyses in the case of **10** are complicated by hydrolysed **10**, which also fluoresces. The background rate of hydrolysis at pH 7.5 and 37°C is usually low, so that the increase in fluorescence is negligible. In the presence of some enzymes, including hβ1β1ADH and *Tb*ADH, however, the hydrolysis rate increased significantly. Although this background was subtracted to calculate the rates shown in Tables 3 and 4, the apparent activity may just be due to error in the measured hydrolysis rate.

Of the other dehydrogenases more distantly related to HLADH which were tested for activity (Table 4), only slSDH and *Lm*G6PDH were active using CL4 as coenzyme. slSDH is another tetrameric member of the long chain alcohol/polyol dehydrogenase family, with subunits showing 25% sequence identity to HLADH [21]. The activity of slSDH using CL4 as coenzyme was 0.55% of the activity using NAD^+ as coenzyme. The coenzyme binding site of slSDH differs from that of HLADH. Only residues Ser-48, His-51 and Asp-223 are conserved. The largest difference is the substitution of glycine for Arg-47. This substitution weakens the binding of the pyrophosphate moiety of NAD^+ in a similar manner to the substitution of histidine for Arg-47 in hβ2β2 ADH [21]. The enzyme exhibiting highest activity using CL4 as coen-

zyme was *Lm*G6PDH, which also had the highest activity using NAD^+ as coenzyme. The activity with CL4 was 0.013% of that with NAD^+. *Lm*G6PDH is a dimer of molecular weight 104,000 [24], and one of the few enzymes to function approximately equally well with either NAD^+ or $NADP^+$ as coenzyme. The kinetics with $NADP^+$ (the marginally preferred coenzyme) and NAD^+ are consistent with the two coenzymes binding to different conformations of the enzyme [25]. ^{1}H NMR studies suggest that there are also significant differences between the conformation of the bound NAD^+ and $NADP^+$ [26]. The versatility of the coenzyme binding site probably accounts for why the enzyme is also functional with CL4.

13 appeared to be coenzymically active with both slSDH and *Lm*G6PDH, and also with *Bm*GluDH and phIDH. *Bm*GluDH belongs to the family of short-chain, non metal-dependent dehydrogenases [27]. The enzyme will utilise either NAD^+ or $NADP^+$ as coenzyme, but $NADP^+$ is preferred [28]. Again this suggests a versatile, accommodating coenzyme binding site. The activity of phIDH is of interest because like *Tb*ADH it is NADP(H)-specific, and not active with NAD^+. No structure for a eukaryotic isocitrate dehydrogenase has been determined, although ^{1}H NMR nuclear Overhauser effect (NOE) measurements suggest that $NADP^+$ is bound with the nicotinamide and nicotinamide–ribose groups in an anti conformation (similarly to HLADH) [29], and that the amide group of the nicotinamide is necessary for the correct positioning and activity of the coenzyme [30].

10 appeared to be slightly active with both *Lm*G6PDH and also with *Bm*GluDH. The activity with slSDH was more convincing, but still calculated to be significantly less than that of CL4 or **13**.

Activity was sought from HLADH and some other dehydrogenases at pH 7.5 and 37°C using the remaining coenzyme analogues (Table 5). For the analogues whose chemically reduced forms had been shown to fluoresce similarly to chemically reduced CL4, the same fluorescence method used for CL4 was used to detect activity. For the remaining analogues, the Roche Cobas Fara assay was adapted to measure absorption. The difference absorption maxima of reduced **6**, **7**, **8** and **11** lie at 470, 490, 510 and 370 nm, respectively [6]. For convenience, however, the interaction of the enzymes with **7** and **8** was observed at 500 nm, and with **6** and **11** was observed at 400 nm.

The results confirmed that none of the remaining coenzyme analogues was coenzymically active with HLADH, but activity was detected with slSDH using **4** and **12**, *Bm*GluDH using **1** and **4**, and with *Lm*G6PDH using 1. The activity of **4** and **12** with slSDH is in contrast to their inability to function coenzymically with HLADH. Thus, slSDH appears to

Table 5
Qualitative screen for activity of various dehydrogenases with different coenzyme analogues, measured by fluorescence or absorption spectroscopy with 200 μM coenzyme at pH 7.5 and 37°C

Enzyme	[Enzyme] (μg ml^{-1})	Substrate	1	2	3	4	5	6	7	8	11	12
HLADH	160	50 mM ethanol	−	−	−	−	−	−	−	−	−	−
HLADH	160	50 mM butan-1-ol	−	−	−	−	−	−	−	−	−	−
*Bm*GluDH	23	50 mM glucose	+	−	−	+	−	−	−	−	−	−
*Lm*G6PDH	58	5.0 mM G6P[a]	+	−	−	−	−	−	−	−	−	−
*Cs*GlyDH	132	100 mM glycerol	−	−	−	−	−	−	−	−	−	−
slSDH	228	100 mM sorbitol	−	−	−	+	−	−	−	−	−	+

+ = change detected in fluorescence/absorption relative to water control.
− = no change detected.
[a] Glucose-6-phosphate.

accept a wider range of artificial coenzymes than HLADH.

4. Summary

The interactions of CL4 and of the analogues **1–13** with HLADH and other dehydrogenases have been compared to the natural coenzymes $NAD(P)^+$. Like CL4, **10** and **13** were coenzymically active in the oxidation of butan-1-ol by HLADH. However, there is a problem with the hydrolysis of **10** which means it is probably an impractical coenzyme. **13** appeared to be more active coenzymically in the oxidation of butan-1-ol than CL4, while **12** was completely inactive. This difference cannot be due to any difference in the inherent ability of the nicotinamide ring to be reduced. Thus, the two sulphonates in the diphenylamino disulphonic acid backbone of CL4 (which are common to Blue N-3 and Nap 1), and their positions, appear to be crucial for coenzymic activity. The increased V_{max} in the oxidation of butan-1-ol by HLADH using **13** as coenzyme may be due to a slightly improved orientation of the molecule in the active site, thereby facilitating hydrogen transfer. We believe that the rate-limiting step for the oxidation of alcohols using the artificial coenzymes may be the transfer of hydrogen, in contrast to the Theorell–Chance mechanism where the dissociation of coenzyme is the rate-limiting step. All of the alcohols oxidised using CL4 as coenzyme are oxidised faster using **13** as coenzyme. In addition, **13** is coenzymically active in the oxidation of ethanol and secondary alcohols. This suggests that the substrate binding site in the HLADH · CL14 complex is structurally more similar to the HLADH · NAD^+ complex.

13 and other analogues are also coenzymically active with hβ1β1ADH, hβ3β3ADH, slSDH, *Bm*GluDH, *Lm*G6PDH and phIDH. These represent a range of dehydrogenases some closely and some only distantly related to HLADH. Thus, the applications of this novel class of compounds are not limited to just one enzyme system. However, more work is required to develop artificial coenzymes with higher activities.

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