

Characterisation of the artificial coenzyme CL4

Richard J. Ansell^{a,1}, David A.P. Small^b, Christopher R. Lowe^{a,*}

^a Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QT, UK

^b Zeneca Bioproducts, PO Box 2, Belasis Avenue, Billingham, Cleveland TS23 31YN, UK

Abstract

A number of NAD⁺ analogues based on the structures of triazine dyes have previously been shown to function coenzymically in the oxidation of alcohols by horse liver alcohol dehydrogenase (HLADH). The simplest of these nicotinamide-containing synthetic compounds is the artificial coenzyme CL4, which has been studied in detail in the present work. The structures of the oxidised and reduced forms of CL4 have been determined by ¹H NMR; the reduced form appears to be the 1,4-dihydronicotinamide, similarly to NADH. CL4 has been shown to be stable across a range of pH values from 3 to 9, and the oxidised form has been regenerated from the reduced form using phenazine methosulphate. The spectrophotometric and fluorometric properties of CL4 have been investigated: It has been possible to develop a real-time fluorescence assay for following the enzymic reduction. This assay was used to show that the oxidation of butan-1-ol by HLADH using CL4 as coenzyme proceeds faster with increasing pH and temperature.

Keywords: Biomimetic coenzyme; Coenzyme analogue; Horse liver alcohol dehydrogenase

1. Introduction

The biomimetic coenzyme Blue N-3 has been shown to bind to the enzyme horse liver alcohol dehydrogenase (HLADH) at the same site as the natural coenzyme nicotinamide adenine dinucleotide (NAD⁺) and to participate in the enzyme-catalysed oxidation of alcohols to aldehydes, being itself converted to a new (reduced) compound [1,2]. The enzyme has been shown to exhibit turnover, so that the reduced compound must be subsequently released. A number of analogues have also been shown to be coenzymically active [3,4], of which the best, Nap 1,

exhibited a k_{cat} for the oxidation of butan-1-ol by HLADH at pH = 9.0 and 25°C which was 9% of that for NAD⁺.

However, the chemical structures of Blue N-3 and Nap 1 have not been conclusively proven, and even less is known about the enzymically reduced forms of these compounds.

The coenzyme analogue CL4 was chosen for further study because it resulted from the shortest synthesis and its putative structure was the simplest of any of the coenzyme analogues thus far shown to exhibit activity. The structure of CL4 (Fig. 2) is identical to those of Blue N-3 and Nap 1 except at the end distal from the nicotinamide moiety. Where Blue N-3 and Nap 1 have anthraquinone and naphthalene groups, respectively, CL4 has a simple acetamido functionality. By synthesising CL4 from its most

* Corresponding author.

¹ Present address: Pure and Applied Biochemistry, University of Lund Chemical Centre, PO Box 124, S22 100 Lund, Sweden.

elementary constituents, and fully characterising the products at each stage of the synthesis, it was hoped that the structure of this, simpler coenzyme, could be proven unambiguously. By isolating sufficient enzymically reduced CL4 to analyze by ^1H NMR it was hoped this compound could be demonstrated to be the 1,4-dihydronicotinamide form of CL4.

Investigations of the biochemistry of Blue N-3 and Nap 1 have been hindered by the absorption of these compounds at 340 nm [1–3]. The absorption at 340 nm and higher wavelengths is attributed to the possibilities for extended electron delocalisation over the entire molecule. In NAD^+ this does not occur and the absorption at 340 nm is negligible. However, NADH absorbs strongly ($\epsilon_{\text{M}}\text{NADH}_{340\text{nm}} = 6220 \text{ l mol}^{-1} \text{ cm}^{-1}$) providing a convenient method for monitoring the conversion of NAD^+ to NADH in real time. Although the reduced forms of Blue N-3 and Nap 1 absorb more strongly at 340 nm than the native compounds, the background absorption of the native compounds at this wavelength is in both cases too high for a spectrophotometer to be able to quantify the change reliably.

CL4 lacks a terminal aromatic group like the anthraquinone of Blue N-3 or the naphthalene of Nap 1, and has been shown to have a much lower absorption at 340 nm [4]. It was hoped this would allow the possibility of a spectrophotometric or fluorometric assay to follow the conversion of CL4 to its reduced form. This would enable a more detailed study of the biochemistry of CL4 in its reaction with HLADH.

2. Experimental

2.1. Materials

2-Chloro-5-nitrobenzenesulphonic acid was supplied by ICI Fine Chemicals Service. All other reagents were analytical grade and were purchased from Sigma Chemical, Fisons PLC, BDH (Merck), or from Aldrich Chemical.

Horse liver alcohol dehydrogenase (HLADH) was purchased from Sigma. Enzyme solutions were prepared and concentrations measured as described previously [4].

2.2. Analytical techniques

Ascending thin-layer chromatography (TLC) was performed with 0.2 mm silica foils (Merck) using a butan-1-ol/propan-2-ol/ethyl acetate/water (2:4:1:3 v/v/v/v) solvent.

High performance liquid chromatography (HPLC) system A was used for the identification of reactants, intermediates and products in the synthesis of the coenzyme mimics, system B for the separation of native and reduced CL4. The elution strategies used were 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 nm or 340 nm.

HPLC system A; a Gilson gradient HPLC system comprising: Model 302 pumps with 5 ml analytical pump heads, model 802 pressure sensing module, dual stage mixer, Model 231 programmable injector with a Model 401 dilutor, variable wavelength Holochrome detector, column oven maintained at 40°C and a Zorbax 5 μm octadecyl (C_{18}) silane column (250 mm \times 4.6 mm) with a 20 mm \times 2 mm pre-column; data were processed by Gilson 712 HPLC System Controller software run on an IBM PS/2 Model 30. The column was equilibrated with methanol – 0.1% (w/v) aqueous *N*-cetyltrimethyl ammonium bromide (70:30, v/v) for several hours before use at a flow rate of 0.5 ml min^{-1} . Injected samples (20 μl) were eluted at 1.0 ml min^{-1} using the following gradient profile: time 0 min, aqueous solvent 30%; time 15.0 min, 10%; time 17.0 min, 0%; time 20.0 min, 0%; time 20.5 min, 30%; time 30.0 min, 30%.

HPLC system B; a Hewlett-Packard gradient HPLC system comprising: 1050 series quaternary pump with column oven maintained at 40°C, 1050 series multiple wavelength detector, 1050 series 21 vial autosampler and a Zorbax 5

μm octadecyl (C_{18}) silane column (250 mm \times 4.6 mm) with a 20 mm \times 2 mm pre-column; data were processed by Hewlett-Packard Chemstation software run 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, 30%; time 10.0 min, 10%; time 10.1 min, 0%; time 14.0 min, 0%; time 14.1 min, 30%; time 22.0 min, 30%.

All UV-visible spectroscopy was performed using a Shimadzu UV-2101PC spectrophotometer equipped with a cell changing device and a peltier temperature control unit. Fluorescence spectroscopy was performed using a Perkin Elmer LS-3 spectrofluorophotometer, the temperature being controlled by a circulating water bath. Rates were recorded using a Kipp and Zonen BD40 chart recorder.

2.3. Characterisation of reactants and products

^1H NMR, elemental analysis and mass spectrometry were performed as a service by the Department of Chemistry, University of Cambridge (part of the Cambridge Centre for Molecular Recognition). ^1H NMR was performed on a Bruker WP 250 MHz instrument. Elemental analysis was performed using a Carlo Erba Elemental Analyzer model 1106. Mass spectrometry was performed by negative ion fast atom bombardment (-ve FAB) from a *m*-nitrobenzyl alcohol matrix with a caesium ion gun (35 keV) on a Kratos MF-890 mass spectrometer.

2.4. 4-Nitro-4'-aminodiphenylamino-2,3'-disulphonic acid

Solid calcium carbonate (25.0 g, 0.25 mol) was added over 5 min to 2-chloro-5-nitrobenzenesulphonic acid (potassium salt, 34.35 g, 0.125 mol) and 2,5-diaminobenzene sulphonic acid (25.0 g, 0.133 mol) in distilled water (125

ml). The slurry was heated cautiously over 30 min to 90°C (1 ml butan-1-ol added to prevent froth), and stirred for 16 h. More water was then added at 90°C until the product was in solution whence it was filtered hot, acidified (12 M HCl, 10 ml), cooled to 5°C and stirred for several hours then filtered. The precipitate was stirred with potassium chloride solution (30% w/v, 30 ml), refiltered and dried under vacuum. Yield: 96%. ^1H NMR (d_6 -dimethylsulphoxide); 7.21 (d, H6, $J = 9.2$), 7.31 (d, H5', $J = 8.7$), 7.35 (dd, H5', $J = 8.5, 2.5$), 7.51 (d, H2', $J = 2.1$), 8.11 (dd, H5, $J = 9.1, 2.8$), 8.45 (d, H3, $J = 2.8$), 9.35 (s, secondary amine).

2.5. 4,4'-Diaminodiphenylamino-2,3'-disulphonic acid

4-Nitro-4'-aminodiphenylamino-2,3'-disulphonic acid (23.0 g, 0.059 mol) in distilled water (500 ml) was dissolved by raising the pH to 7 (5 M KOH), then reduced by catalytic hydrogenation using palladium on activated charcoal (10% w/w, approximately 200 mg) at atmospheric pressure. After 24 h, reduction was observed to be complete by TLC and the solution was filtered hot to remove the catalyst, reduced in volume to less than 100 ml using a high vacuum rotary evaporator, cooled to 5°C and acidified to pH = 2 (12 M HCl). The precipitate was filtered, washed with potassium chloride solution (30% w/v) and dried under vacuum. Yield: 85%. ^1H NMR (d_6 -dimethylsulphoxide); 7.15 (dd, H6', $J = 8.5, 2.5$), 7.24 (d, H5', $J = 8.4$), 7.26 (dd, H5, $J = 9.0, 2.5$), 7.31 (d, H6, $J = 8.6$), 7.44 (d, H2', $J = 2.6$), 7.70 (d, H3, $J = 2.5$), 8.7 (br s, secondary amine).

2.6. 4-Acetamido-4'-aminodiphenylamino-2,3'-disulphonic acid

A solution of 4,4'-diaminodiphenylamino-2,3'-disulphonic acid (8.98 g, 0.025 mol) in water (100 ml) was adjusted to pH = 7.0 (1.0 M LiOH) and stirred at 5°C. Acetic anhydride (3

ml, 0.032 mol) was added dropwise to the solution, which was then stirred for 1 h. The reaction was shown to be complete by analytical TLC whence the solution was acidified to pH = 1.5 (12 M HCl) and filtered. The precipitate was washed with HCl solution (1 M, 20 ml), made into a slurry with acetone, refiltered, washed again with acetone and dried under vacuum. Yield = 76%. ^1H NMR (d_6 -dimethylsulphoxide); 2.99 (3Hs, Me-), 7.57 (dd, H6', $J = 8.6$, 2.6), 7.69 (d, H6, $J = 8.6$), 7.69 (d, H5', $J = 7.1$), 7.84 (d, H2', $J = 2.6$), 8.09 (dd, H5, $J = 8.8$, 2.5), 8.35 (d, H3, $J = 2.4$), 10.40 (s, amide NH-).

2.7. *1-(4'-(4''-Acetamido-2''-sulphoanilino)-2'-sulphoanilino)-3-amino-5-chloro-2,4,6-sym-triazine*

Cyanuric chloride (3.53 g, 0.019 mol) was dissolved in acetone (10 ml) and stirred in distilled water (50 ml) at 5°C. 4-Acetamido-4'-aminodiphenylamino-2,3'-disulphonic acid (7.70 g, 0.019 mol) in distilled water (30 ml) was adjusted to pH = 7.0 (1.0 M KOH) and added dropwise to the cyanuric chloride over 20 min. During this time and while the solution stirred for a further 3.5 h, the pH was maintained between 6–7 by simultaneous addition of KOH solution (first 1.0 M then 0.2 M) using a pHstat (VIT90 Video Titrator, Radiometer Copenhagen). The reaction was determined complete by analytical TLC whence ammonia solution (14.8 M, 20 ml) was added to the reaction mixture, the temperature raised to 20°C, and stirring continued for a further 6 h. When no further change was observed by analytical TLC impurities were precipitated by addition of potassium chloride solution (20% w/v, 5 ml) and HCl solution (12 M, to pH = 6.5), and removed by filtration. The solution was further acidified to pH = 1.5 (12 M HCl), and left overnight at 5°C. The precipitate was then filtered, made into a slurry with acetone, refiltered and dried under vacuum. Yield: 36%. ^1H NMR (d_6 -dimethylsulphoxide); 2.03 (3Hs, Me-), 7.01 (dd, H5', $J = 8.6$, 2.9), 7.11 (d, H6', $J = 8.7$),

7.40 (d, H3', $J = 2.8$), 7.61 (d, H6'), 7.61 (dd, H5', $J = 8.8$, 2.5), 7.86 (d, H3', $J = 2.5$), 9.87 (s, amide NH-), 9.94 (s, secondary amine).

2.8. *1-(4'-(4''-Acetamido-2''-sulphoanilino)-2'-sulphoanilino)-3-amino-5-(4' carboxamido pyridino)-2,4,6-sym-triazine (CL4)*

1-(4'-(4''-Acetamido-2''-sulphoanilino)-2'-sulphoanilino)-3-amino-5-chloro-2,4,6-sym-triazine (4.20 g, 7.93 mmol) and nicotinamide (2.91 g, 23.80 mmol) in distilled water (30 ml) were stirred at 90°C for 5 h during which time the colour changed to dark red/brown. The solution was cooled to 5°C and potassium chloride solution (20% w/v, 10 ml) added. The precipitate was filtered, made into a slurry with acetone, refiltered and dried under vacuum. Yield: 40%. ^1H NMR (d_6 -dimethylsulphoxide); 2.01 (3Hs, Me-), 7.08 (dd, H5', $J = 7.8$), 7.15 (d, H6', $J = 8.6$), 7.43 (d, H3', $J = 1.9$), 7.56 (dd, H5', $J = 7.8$), 7.83 (d, H3', $J = 1.8$), 8.07 (d, H6', $J = 8.3$), 8.26 (s, secondary amine), 8.39 (dd, Hnic5, $J = 7.3$), 8.83 (s, secondary amine), 9.29 (d, Hnic4, $J = 7.8$), 9.86 (s, amide NH-), 9.90 (d, Hnic6, $J = 5.9$), 10.16 (s, Hnic2). MS (m/e): 727.5 ($\text{C}_{23}\text{H}_{20}\text{N}_9\text{S}_2\text{O}_8\text{K}_2\text{Cl}$), 691.4 ($\text{C}_{23}\text{H}_{20}\text{N}_9\text{S}_2\text{O}_8\text{K}_2$), 669.3 ($\text{C}_{23}\text{H}_{20}\text{N}_9\text{S}_2\text{O}_8\text{K}\cdot\text{OH}$), 653.2 ($\text{C}_{23}\text{H}_{20}\text{N}_9\text{S}_2\text{O}_8\text{K}$), 614.2 ($\text{C}_{23}\text{H}_{20}\text{N}_9\text{S}_2\text{O}_8$), 533.3 ($\text{C}_{17}\text{H}_{16}\text{N}_7\text{S}_2\text{O}_7\text{K}$), 494.3 ($\text{C}_{17}\text{H}_{16}\text{N}_7\text{S}_2\text{O}_7$), 438.2 (MeCONH(C₆H₄SO₃)NH (C₆H₄SO₃)NH₂K), 380.1 ((C₆H₄SO₃)NH(C₆H₄SO₃)NH₂K), 341.1 ((C₆H₄SO₃)NH(C₆H₄SO₃)NH₂), 227.2 (MeCON(C₆H₄SO₃)NH), 188.2 (H₂N(C₆H₄SO₃)NH₃).

Crude CL4 was purified by Sephadex LH-20 column chromatography as described previously [4]. The resulting CL4 was 100% pure as determined by HPLC, and the solubility in water was at least 10 mg ml⁻¹ compared to just 2 mg ml⁻¹ for the crude material.

The extinction coefficient of CL4 was determined as described previously [4], except that the molecular weight calculated from elemental

analysis data was used instead of the theoretical molecular weight, to calculate the concentration of the CL4 stock solution.

2.9. pH stability of CL4

To determine whether CL4 is stable over a wide pH range, 2 mM CL4 was incubated at 30°C for 1 h in potassium citrate (10 mM)-potassium phosphate (10 mM)-glycine (10 mM) buffer at each of the following pH values: pH = 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0. The samples were subsequently analyzed by HPLC.

2.10. Electrochemical properties of CL4

Dc cyclic voltammetry experiments were performed with a 3-electrode water jacketed glass cell with a working volume of 1 ml. The reference and counter electrodes were a calomel electrode and platinum wire respectively. The working electrode used for all experiments was a glassy carbon electrode (3 cm × 3 mm diameter). The background electrolyte was 100 mM sodium phosphate buffer pH = 7.50. A Princeton Applied Research Model 273 potentiostat/galvanostat was used in conjunction with a Gould series 6000XYt recorder. The temperature of the cell was maintained at 25°C by means of a Julabo F10 water bath and circulator during all experiments. The glassy carbon electrode was pre-treated [5] by sweeping between +1.80 V and –2.00 V at a scan rate of 0.1 V s⁻¹, while stirring for 20 min in the background buffer solution. The background cyclic voltammograms were recorded until successive cycles yielded a superimposable trace (about 15 min). A 1 mM solution of CL4 in 100 mM sodium phosphate pH = 7.50 was placed in the cell and the voltammetric curve recorded. The experiment was repeated with 1 mM Nap 1, 1 mM NAD⁺, and 1 mM nicotinamide. Between experiments on different solutions the electrodes were washed in distilled water and the pre-treatment repeated.

2.11. Isolation of reduced CL4 for ¹H NMR

HLADH (12.8 μM), CL4 (2 mM), butan-1-ol (100 mM), sodium phosphate buffer pH = 7.5 (95 mM) in 10 ml were incubated for 16 h at 35°C, whereupon HPLC analysis showed reduction of CL4 to be essentially complete. The mixture was lyophilised, resuspended in methanol/water (1:1 v/v, 5 ml), filtered through a 0.45 μm filter, then purified by Sephadex LH-20 column chromatography as described previously [4]. Yield: 4.0 mg, 33%. ¹H NMR (d₆-dimethylsulphoxide); 1.99 (3Hs, Me–), 5.15 (d, Hnic5, *J* = 8.6), 6.98 (dd, H5', *J* = 8.8), 7.05 (s, secondary amine), 7.07 (d, H6', *J* = 8.7), 7.07 (d, H6', *J* = 8.7), 7.29 (d, Hnic6, *J* = 8.4), 7.39 (dd, H3', *J* = 2.7), 7.50 (dd, H5', *J* = 8.7, 2.6), 7.79 (d, H3', *J* = 2.6), 8.22 (s, secondary amine), 8.27 (2Hs, Hnic4), 9.56 (s, Hnic2), 9.80 (s, amide NH–).

2.12. Reoxidation of enzymically reduced CL4 by phenazine methosulphate

HLADH (55.3 μM), CL4 (1.0 mM), butan-1-ol (1.0 mM), sodium phosphate buffer pH = 7.5 (85.3 mM) in 2 ml were incubated for 16 h at 35°C, whereupon HPLC analysis showed reduction of CL4 to have essentially ceased. The reaction mixture was centrifuged at 13000 rpm in an MSE Microcentaur microcentrifuge to remove denatured protein.

To 200 μl of this crude preparation of reduced CL4 were added an equal volume of an aqueous solution of phenazine methosulphate (PMS) (2.0 mM). After 10 min, 10 μl were analyzed by HPLC. A control to which only water was added was also analyzed by HPLC, and a 1.0 mM solution of PMS.

2.13. UV-visible absorption spectra of CL4 and chemically reduced CL4

CL4 (80 μM) and sodium borohydride (800 μM) in 500 μl were incubated for 30 min at

20°C, whence HPLC showed complete conversion of CL4 to a new species. The absorption spectrum of the reduced CL4 was recorded in a 10 mm pathlength silica cuvette against a distilled water blank and compared to a control in which sodium borohydride was absent, shown by HPLC to contain only native CL4.

2.14. UV-visible absorption and fluorescence spectra of CL4 and enzymically reduced CL4

HLADH (30.3 μM), CL4 (1.0 mM), butan-1-ol (100 mM), sodium phosphate buffer pH = 7.5 (73.1 mM) in 1.5 ml were incubated at 37°C. When reduction of CL4 was shown by HPLC to be 87% complete, 40 μl were withdrawn, added to 460 μl distilled water in a 10 mm pathlength silica cuvette and the absorption spectrum recorded. The spectrum was compared to a control in which butan-1-ol was omitted, shown to contain only native CL4 by HPLC.

The fluorescence emission spectra for native and reduced CL4 in the presence of an excitation at 350 nm were then recorded in 10 mm square disposable cuvettes.

2.15. Spectrophotometric demonstration of the coenzymic activity of CL4 in the oxidation of butan-1-ol by HLADH

In a 1 mm pathlength quartz cuvette were HLADH (34.0 μM), CL4 (500 μM), butan-1-ol (100 mM), sodium phosphate (91.6 mM) buffer pH = 7.5 in a total volume of 200 μl . HLADH, CL4 and buffer were pre-equilibrated for 5 min at 37°C. Butan-1-ol was added to initiate the reaction and the absorption at 350 nm followed and compared to a control to which water was added in place of butan-1-ol. The first order rates dA_{350}/dt were calculated by the software. The procedure was repeated for a control which contained no CL4, and for a control which contained no HLADH.

2.16. Fluorometric demonstration of the coenzymic activity of CL4 in the oxidation of butan-1-ol by HLADH

In a total volume of 2 ml in a disposable 10 mm square cuvette were HLADH (10 μM), CL4 (100 μM), butan-1-ol (100 mM), sodium phosphate (86.9 mM) buffer pH = 7.5.

HLADH, CL4 and buffer were pre-equilibrated at 35°C for 5 min while the fluorescence (excitation 350 nm, emission 450 nm) was observed to be constant. Butan-1-ol was added to initiate the reaction and the increase in fluorescence recorded. To confirm that the rate of reaction was dependent on the concentration of enzyme and of butan-1-ol, the experiment was repeated using 20 mM butan-1-ol, then using 4.0 μM HLADH.

2.17. pH optimum for the oxidation of butan-1-ol by HLADH using CL4 as coenzyme

Potassium citrate (10 mM)-potassium phosphate (10 mM)-glycine (10 mM) buffer was prepared at each of the following pH values; 5.0, 6.0, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5. In a total volume of 2 ml in a disposable fluorometer cuvette were HLADH (5 μM), CL4 (100 μM), potassium citrate (8.09 mM)-potassium phosphate (8.09 mM)-glycine (8.09 mM) buffer at each pH. The actual pH of the reaction mixture was recorded and it was pre-equilibrated at 25°C for 5 min while the fluorescence (excitation 350 nm, emission 450 nm) was observed to be constant. Butan-1-ol (100 mM) was added and the increase in fluorescence recorded.

2.18. Temperature optimum for the oxidation of butan-1-ol by HLADH using CL4 as coenzyme

The assay mixture was as above, using the pH = 9.0 buffer. The mixture was pre-equilibrated at 20°C for 5 min while the fluorescence was observed to be constant. Butan-1-ol (100 mM) was added and the increase in fluo-

rescence recorded. The assay was repeated at 25°C, 30°C, 35°C, 37°C and 40°C, allowing the system to stabilise at the new temperature between each assay.

2.19. Reduction of butan-1-al by HLADH using reduced CL4 as coenzyme

A crude sample of reduced CL4 was prepared as before. To 200 μ l of this mixture were added HLADH and butan-1-al. In a total volume of 400 μ l were HLADH (32.3 μ M), reduced CL4 (500 μ M), butan-1-al (55 mM). The reaction mixture was incubated at 37°C and aliquots were withdrawn after 30 min and 12 h and analyzed by HPLC. The experiment was repeated using acetaldehyde (54 mM and 179 mM) in place of butan-1-al, and in the absence of substrate.

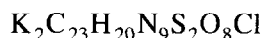
3. Results and discussion

3.1. Synthesis of CL4

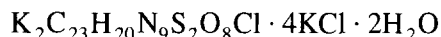
CL4 was synthesised essentially as previously [4], except the synthesis of 4,4'-diaminodiphenylamino-2,3'-disulphonic acid, and that intermediates and products were characterised by ^1H NMR. The chemical shifts assigned to the protons in these compounds are in full agreement with the expected *ortho/para* shielding effects of amine groups and deshielding effects of sulphonate and nitro groups.

The experimental data for the elemental analysis of CL4 do not correspond well with the theoretical values. However, with the method of isolation taken into account, the experimental values may be accounted for. Since CL4 was precipitated with potassium chloride at low pH, salt and water may be added to the molecular formula. Assuming the two sulphonates to be balanced by potassium ions and the quaternary pyridinium by a chloride ion, the model requires a further 2 equivalents of water and 4 equiva-

lents of potassium chloride, giving an apparent molecular mass of 1062.5;



$$M_r = 728.2; \text{C } 37.90\% \text{ H } 2.77\% \text{ N } 17.31\%$$



$$M_r = 1062.5; \text{C } 26.00\% \text{ H } 2.28\% \text{ N } 11.86\%$$

Found; C 26.12% H 2.39% N 11.97%

The major ion peaks in the negative ion fast atom bombardment mass spectroscopy spectrum could largely be assigned to logical CL4 fragments and the fragments of higher molecular mass supported the theory that potassium chloride remained present in the sample in significant quantities.

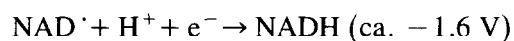
The calculated M_r of 1062.5 was used to determine the molar extinction coefficient at the λ_{max} (318 nm). The value obtained ($\epsilon_{318\text{nm}} = 24 \text{ mM}^{-1} \text{ cm}^{-1}$) was used to calculate the CL4 concentration in all subsequent experiments. The phenomenon of stacking, which gives rise to a change in the apparent extinction coefficient of dyes such as C.I. Reactive Blue 2 at concentrations above 12 μ M [6], is not evident with CL4, which is more hydrophilic.

3.2. pH stability

CL4 was shown to be stable at 30°C in the range pH = 3–9 (Fig. 1), but labile at more alkaline pH values. The base lability and acid stability of CL4 are to be expected if its chemistry is analogous to NAD^+ [7]. The alkaline lability of Blue N-3 has been attributed to cleavage of the triazine–nicotinamide bond, so would be expected for CL4. By contrast, Blue N-3 is labile also under acidic conditions.

3.3. Electrochemical properties of CL4

NAD^+ is reduced electrochemically in a two step process [8];



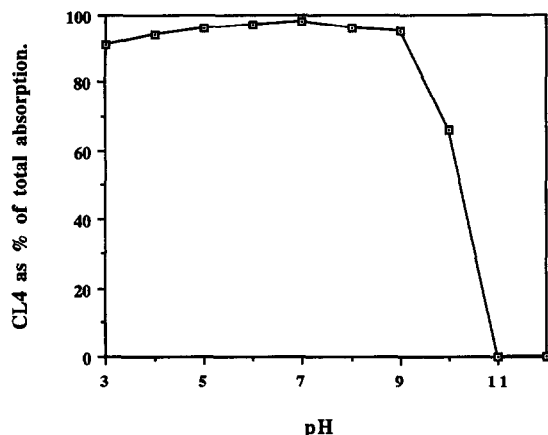


Fig. 1. Stability of CL4 over the pH range. After incubation for 1 h at 30°C, samples were analyzed by HPLC, and the peak due to CL4 calculated as a % of the total absorption.

1,4-Dihyronicotinamide adenine dinucleotide, which is the principal product, is oxidised back to NAD^+ in a single step (at ca. 0.5 V). The values obtained by dc cyclic voltammetry for the first reduction potential of NAD^+ and oxidation potential of NADH were -1.23 V and 0.6 V respectively. CL4 gave values of -0.8 V and $+0.6$ V for the first reduction and oxidation potential respectively (Table 1). The less negative reduction potential of CL4 is due to the greater electron-withdrawing properties of the triazine ring which is coupled to the nicotinamide in CL4 compared to the ribose group of NAD^+ . Thus, CL4 is more easily reduced electrochemically than NAD^+ . Very similar results were obtained with Nap 1, as might be expected since the nicotinamide in Nap 1 is also coupled to a triazine group.

3.4. Characterisation of enzymically reduced CL4

It has been shown previously that the reaction of HLADH, butan-1-ol and CL4 gives rise to a new species which can be separated from CL4 by HPLC, and which is not produced in the absence of any one of the three components [4]. The same has been shown also for Blue N-3 and the other biomimetic coenzymes [1–4]. If

the reaction involves the transfer of a hydride ion from butan-1-ol to CL4, then butan-1-al and reduced CL4 are expected to be the products. In order to confirm the identity of the reduced CL4, the new compound was purified and analyzed by ^1H NMR (Fig. 2). The spectrum of reduced CL4 gives sharper peaks than the spectrum of CL4, probably due to its greater solubility.

The signals attributed to the acetamido and benzenesulphonate protons of CL4 have close equivalents in the spectrum of reduced CL4. All of the signals for reduced CL4 come slightly further upfield, consistent with the nicotinamide ring of CL4 being replaced by an uncharged, therefore more shielding, dihyronicotinamide ring.

The nicotinamide ring of CL4 could be reduced to form a 1,2-, 1,4- or 1,6-dihyronicotinamide ring, which would give different ^1H NMR spectra. The chemical shifts of Hnic2, Hnic4 and Hnic6 are determined by the deshielding effects of the quaternary nitrogen atom and the amide group attached to the ring; thus, and similarly to NADH, Hnic2 is expected to come further downfield than Hnic4 which comes further downfield than Hnic6. In the spectrum of reduced CL4, the signal integrating to 2 H is attributed to Hnic4 because it comes between signals attributed to Hnic6 and Hnic2. The observed shifts and coupling of the other signals are also consistent with reduced CL4 being the 1,4-dihyronicotinamide analogue of CL4 similarly as for NAD(H) [9].

Table 1
Redox potentials of 1 mM NAD^+ , nicotinamide, Nap 1 and CL4 at pH 7.5 and 25°C as measured by dc cyclic voltammetry. Only the first peak observed on each cycle is shown

Coenzyme	Reduction potential (V)	Oxidation potential (V)
NAD^+	-1.23	$+0.60$
Nicotinamide	-1.55	-0.16
Nap 1	-0.92	$+0.40$
CL4	-0.88	$+0.54$

3.5. Chemical regeneration of CL4

The regeneration of nicotinamide cofactors has received much attention [10] as it removes the necessity of using stoichiometric amounts of expensive NAD(H). Although coenzymes based on CL4 would be less expensive than NAD(P), it would still be economically useful if they could be regenerated by an even less expensive chemical reducing or oxidising agent such as phenazine methosulphate (PMS).

PMS has been shown to efficiently reoxidise

both NADH [11] and reduced Blue N-3 [1]. When 1.0 mM phenazine methosulphate was added to a crude preparation of reduced CL4 (0.5 mM), HPLC analysis showed that CL4 was regenerated. Thus, reduced CL4 can be readily reoxidised also.

3.6. UV-visible absorption properties of enzymically reduced CL4

Chemical reduction of the nicotinamide ring of NAD⁺ using sodium borohydride gives rise

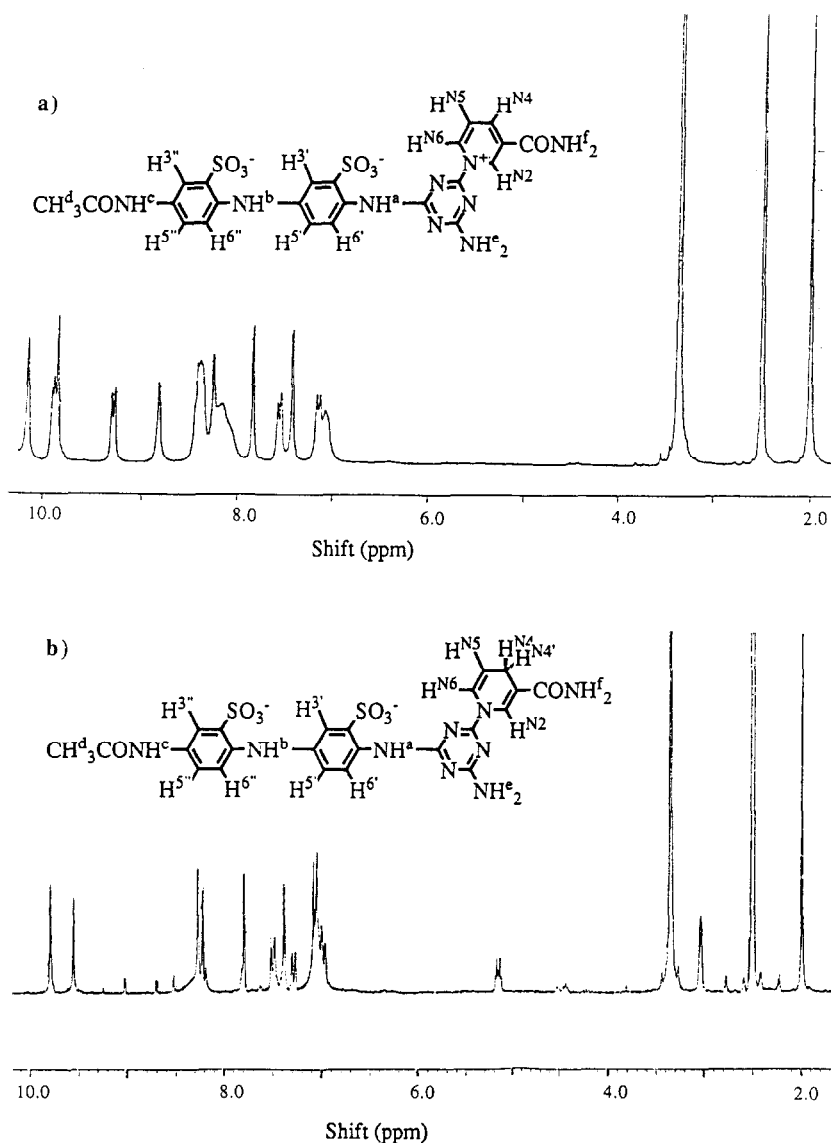


Fig. 2. ¹H NMR spectra (d₆-dimethylsulphoxide) for (a) CL4 and (b) enzymically reduced CL4.

to a mixture of the 1,2-, 1,4- and 1,6-dihydronicotinamide isomers of NADH, which have slightly differing spectra but similar absorption maxima at 340 nm [12]. When sodium borohy-

dride was added to CL4 a new compound was detected by HPLC, having an identical retention time to the product of the reaction of HLADH, butan-1-ol and CL4. This compound was con-

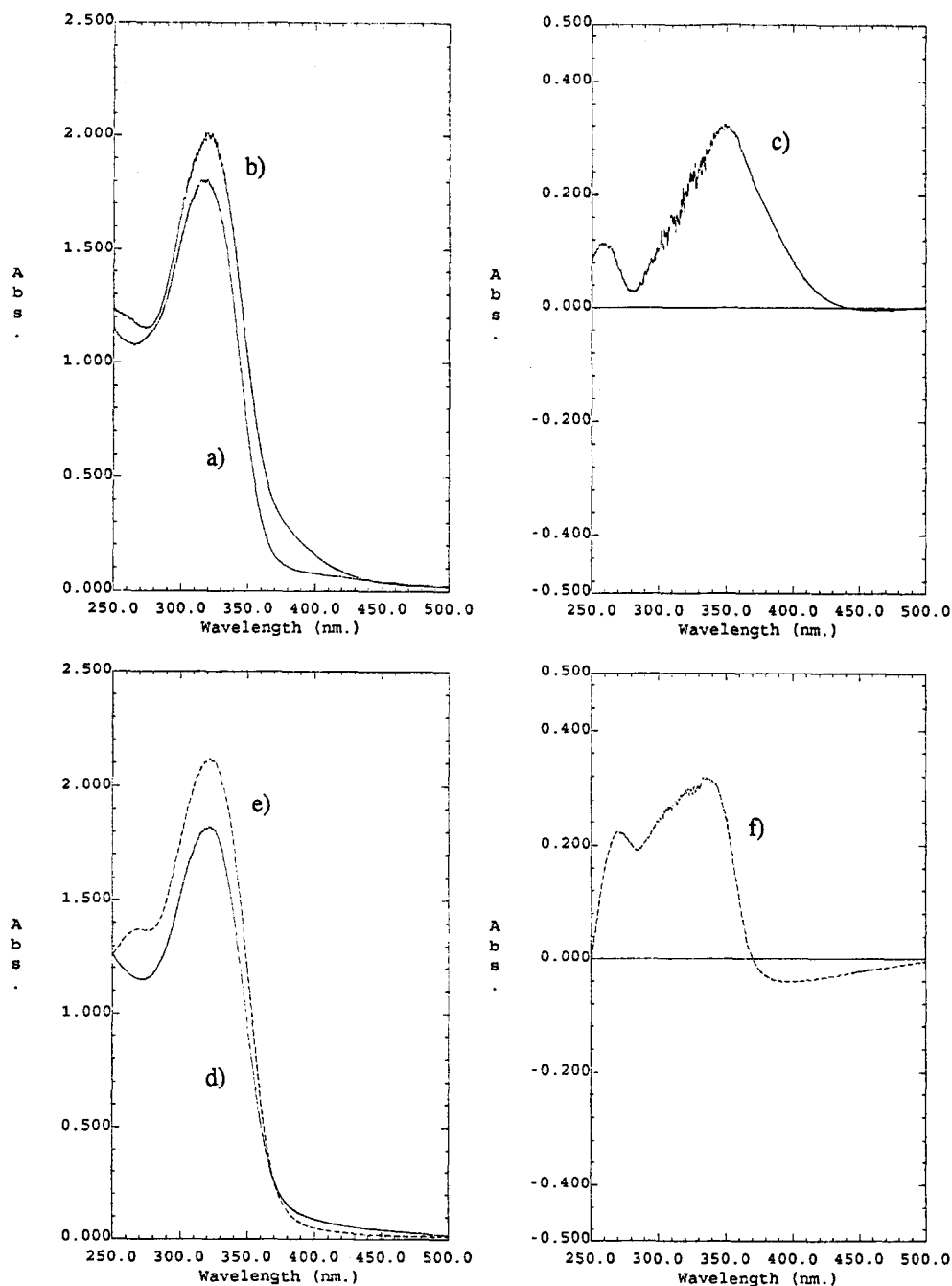


Fig. 3. UV-visible absorption spectra of CL4, chemically and enzymically reduced CL4. (a) 80 μ M CL4. (b) 80 μ M CL4 chemically reduced by sodium borohydride. (c) Difference absorption spectrum generated by chemical reduction. (d) 80 μ M CL4. (e) 80 μ M CL4, 87% enzymically reduced. (f) Difference absorption spectrum generated by enzymic reduction.

cluded to be the 1,2-, 1,4- or 1,6-dihydronicotinamide analogue of CL4, expected to have essentially identical HPLC retention times. The difference spectrum of CL4 and chemically reduced CL4 (Fig. 3) showed a maximum at 350 nm and was observed to be very similar in form to the difference spectrum of Nap 1 and chemically reduced Nap 1 [3].

3.7. UV-visible absorption properties of enzymically reduced CL4

Enzymic reduction of CL4 also gave a difference spectrum with a maximum at about 350 nm (Fig. 3), although the difference in absorption at 270 nm was significantly higher than for chemically reduced CL4. Such a difference may be understood if chemical reduction produces a mixture of 1,2-, 1,4- and 1,6-dihydronicotinamide analogues as with NAD^+ , but enzymic reduction produces only the 1,4- analogue.

The difference in absorption of NAD(P)^+ and NAD(P)H at 340 nm has provided a convenient method for measuring the rates of NAD(P)(H) dependent reactions in real time [13]. As there is a difference in the absorption spectra of oxidised and reduced CL4 at 350 nm,

it was attempted to follow the reduction of CL4 in the reaction with HLADH and butan-1-ol by monitoring absorption at this wavelength.

When HLADH, CL4 and butan-1-ol were incubated at $\text{pH} = 7.5$ and 37°C , there was an increase in absorption at 350 nm of 7.0 ± 0.2 mAU min^{-1} over 10 min. The rate for a control in which water replaced butan-1-ol was 2.7 ± 0.1 mAU min^{-1} . For controls in which HLADH or CL4 were omitted, rates were negligible. Although these results showed that reduction of CL4 can be followed by absorption at 350 nm, the rate was very small, and errors at such high background absorptions (> 1.0 AU) limited the practicable concentration of CL4 to $500 \mu\text{M}$, even using 1 mm pathlength cuvettes. There was also a background rate in the absence of butan-1-ol, possibly due to precipitation of HLADH, which was at a high concentration in the assay ($33.9 \mu\text{M}$), or to a very slow binding process.

3.8. Fluorescence properties of enzymically reduced CL4

Although NAD^+ has no chromophore which gives rise to a fluorescence spectrum, NADH

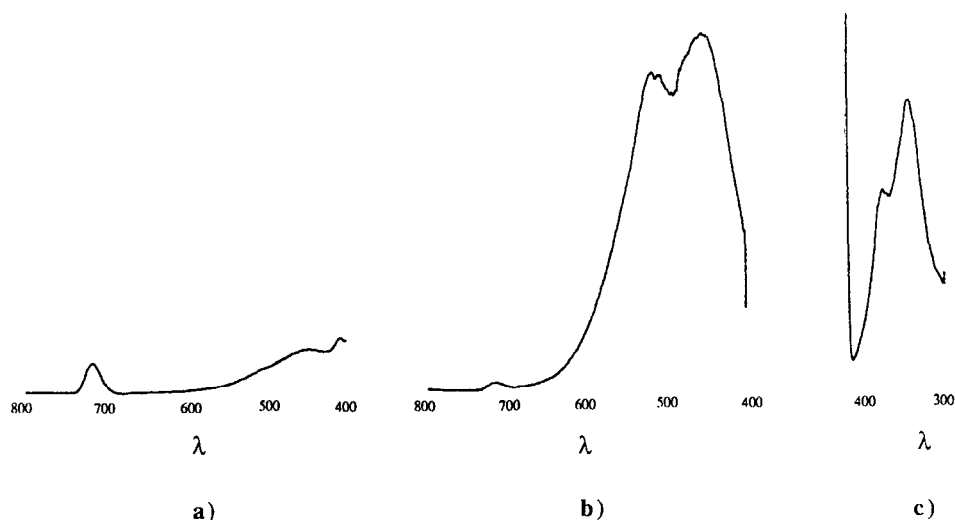


Fig. 4. Fluorescence properties of CL4 and enzymically reduced CL4, $50 \mu\text{M}$ in 100 mM sodium phosphate buffer $\text{pH} = 7.5$. (a) Emission spectrum of CL4 when excited at 350 nm. (b) Emission spectrum of reduced CL4 when excited at 350 nm. (c) Fluorescence of reduced CL4 at 450 nm when the excitation wavelength was varied.

contains the 1,4-dihydronicotinamide chromophore which absorbs at 340 nm and emits fluorescence at 450 nm. Since reduced CL4 contains the same chromophore, it was expected to show a similar fluorescence spectrum.

The emission spectrum for enzymically reduced CL4 in the presence of an excitation at 350 nm showed a maximum at 450 nm (Fig. 4b). Native CL4 did not give this emission maximum, or any other significant emission (Fig. 4a). When the emission wavelength was fixed at 450 nm, a scan of the excitation spectrum of reduced CL4 showed a maximum at 350 nm (Fig. 4c).

Fluorescence assays utilising the native fluorescence of NADH have been employed for a number of years to measure many metabolites using NAD⁺ linked reactions [14]. The technique is much more sensitive than absorption spectroscopy, and, as reduced CL4 fluoresces in much the same way as NADH should provide a more convenient method to follow the reduction of CL4.

When HLADH, CL4 and butan-1-ol were incubated at pH = 7.5 and 37°C, there was an increase in fluorescence (excitation 350 nm, emission 450 nm). There was no detectable rate in the absence of butan-1-ol or CL4. When the assay was repeated with 20 mM butan-1-ol in place of 100 mM, the rate fell from 4.0 FU min⁻¹ (fluorescence units per min) to 3.7 FU min⁻¹. When the assay was repeated with 4.0 μM HLADH in place of 10 μM, the rate fell from 4.0 FU min⁻¹ to 1.7 FU min⁻¹. Thus, fluorescence appears to be a suitable method for the assay of CL4. However, because CL4 contains a chromophore which absorbs at 350 nm, increasing the concentration of CL4 has the effect of decreasing the excitation intensity, such that the emission due to a given amount of reduced CL4 decreases. Thus, the apparent rates at different concentrations of CL4 cannot be directly compared, and above 400 μM CL4, rates are almost impossible to measure. This effect is commonly observed when proteins which fluoresce are titrated with ligands which

absorb at the excitation wavelength, and is known as the 'primary absorption' or 'inner filter' effect [15].

Despite these problems, the fluorescence assay provided a straightforward continuous method for comparing relative rates of reduction of CL4, at the same concentration of CL4, in real time. The assay was used to measure the pH and temperature dependence of the oxidation of butan-1-ol by HLADH using CL4 as coenzyme.

3.9. pH dependence for the oxidation of butan-1-ol by HLADH using CL4 as coenzyme

The oxidation of ethanol by HLADH using NAD⁺ as coenzyme has a maximum at ~ pH = 10 [16]. The pH profile was shown to be consistent with the Theorell–Chance mechanism [17], with the rate determining step (under conditions of saturating NAD⁺ and ethanol) being the dissociation of NADH. Dissociation of NADH was promoted by deprotonation of two groups with pK_a's 6.4 and 9.6.

The pH profile of the rate of butan-1-ol oxidation by HLADH using CL4 as coenzyme determined at 25°C is shown in Fig. 5. At the high concentrations required to observe activity, the enzyme precipitates below pH = 6.5. This

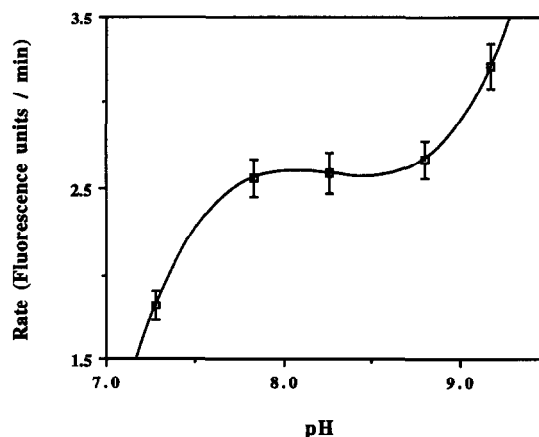


Fig. 5. Dependence on pH of the rate of oxidation of butan-1-ol by HLADH using CL4 as coenzyme, as measured by fluorescence at 25°C. Curve fitted by third-order polynomial regression.

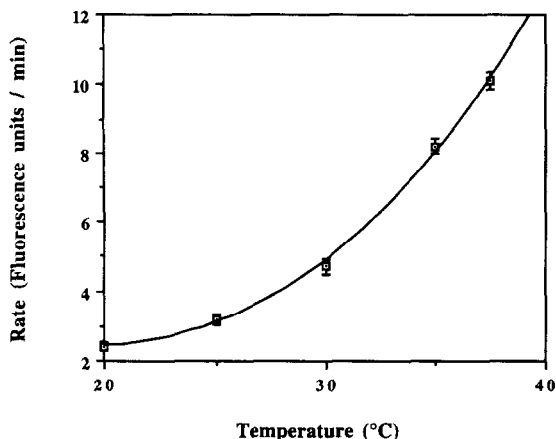


Fig. 6. Dependence on temperature of the rate of oxidation of butan-1-ol by HLADH using CL4 as coenzyme, as measured by fluorescence at pH = 9.0. Curve fitted by third-order polynomial regression.

precipitation gives a high background rate on the fluorometer due to reflected light, so that activity cannot be observed reliably. Above pH = 9.5 CL4 is hydrolysed. The hydrolysis product also fluoresces, so that there is a high background rate at high alkaline pH. Between pH = 7.3 and pH = 9.2, the pH dependence is consistent with two pK_a values of $> \text{pH} = 9.1$ and $< \text{pH} = 7.4$, deprotonation of each group leading to an increase in the observed rate.

3.10. Temperature dependence for the oxidation of butan-1-ol by HLADH using CL4 as coenzyme

The temperature dependence of the rate of butan-1-ol oxidation by HLADH using CL4 as coenzyme determined at pH = 9.0 is shown in Fig. 6. Although the observed reaction rate increases dramatically above 30°C, the background rate also begins to increase due to hydrolysis of CL4, and by 40°C is a significant proportion of the reaction rate. An assay at pH = 7.5 and 37°C gave no background rate, and a reaction rate between that at pH = 7.5 and 25°C and that at pH = 9.0 and 37°C. Thus, conditions of pH = 7.5 and 37°C were chosen for further studies of the coenzymic activity of

CL4 with HLADH, since although these were not the optimal conditions, there was no complication from background hydrolysis of CL4.

3.11. Coenzymic activity of reduced CL4

The reaction of NAD^+ and alcohols catalysed by HLADH is an equilibrium; the reverse reaction of NADH and carbonyls is also observed, and is slightly thermodynamically favoured; the nett effect is that neither reaction will go to completion, an equilibrium mixture is always produced.

With Blue N-3, no reaction of the reduced analogue with acetaldehyde could be detected in the presence of HLADH, and the reaction of Blue N-3 and alcohols was shown to proceed to completion [1]. It was considered that butan-1-al might be a better substrate than acetaldehyde for observing activity in this direction; butan-1-ol has been shown to be a better substrate than ethanol in the forward direction [2]. Reduced CL4 was incubated with HLADH and large excesses of acetaldehyde or butan-1-al in order to determine if the carbonyl compounds could be reduced to the corresponding alcohols. No production of CL4 in the presence of HLADH and carbonyl compounds was observed, consistent with the similar chemical environment of the functional nicotinamide moiety in Blue N-3 and CL4.

4. Summary

The structure of CL4 has been proven, and enzymic reduction has been shown to give rise to the 1,4-dihydronicotinamide form of the coenzyme analogue. In addition to its simpler synthesis, CL4 has several other advantages over the biomimetic coenzymes we have studied previously; it has a higher solubility in water, it is stable over a wider range of pH, and enzymic reduction may be followed by a simple fluorometric assay. This assay has been used to show that the enzymic activity of HLADH with CL4

as coenzyme increases with increasing pH and temperature, similarly as with NAD⁺. The activity remains low compared to NAD⁺, and, unfortunately, activity could not be demonstrated with reduced CL4 in the reduction of aldehydes by HALDH. This may be attributed to the electron-withdrawing nature of the triazine ring linked to the nicotinamide in CL4, Blue N-3 and Nap 1, which has been shown to give these coenzymes different redox-properties from the natural coenzyme.

In future work we shall use fluorescence to investigate the interaction of CL4 with HLADH and other NAD(P)-dependent enzymes in more detail. In addition, the relatively simple structure of CL4 will provide a comparison for the properties of new analogues to be synthesised, which will differ only slightly from CL4 in their chemical structure.

Acknowledgements

We would like to thank the Biotechnology Directorate of the Science and Engineering Research council and Zeneca Bioproducts for their financial support.

References

- [1] S.J. Burton, C.V. Stead, R.J. Ansell and C.R. Lowe, *Enzyme Microb. Technol.* 18 (1996) 570.
- [2] S.B. McLoughlin and C.R. Lowe, *Enzyme Microb. Technol.*, in Press.
- [3] S. Dilmaghanian, C.V. Stead, R.J. Ansell and C.R. Lowe, *Enzyme Microb. Technol.*, in Press.
- [4] R.J. Ansell, S. Dilmaghanian, C.V. Stead and C.R. Lowe, *Enzyme Microb. Technol.*, submitted.
- [5] W.J. Blaedel and R.A. Jenkins, *Anal. Chem.* 47 (1975) 1337.
- [6] S. Subramanian, *Arch. Biochem. Biophys.* 216 (1982) 116.
- [7] N.J. Oppenheimer, in: D. Dolphin, R. Poulson and O. Avramović (Eds.), *Pyridine nucleotide coenzymes, part A*, Wiley, New York, 1987, p. 323.
- [8] J. Moiroux and P.J. Elving, *J. Am. Chem. Soc.* 102 (1980) 6533.
- [9] M.E. Pullman and S.P. Colowick, *J. Biol. Chem.* 206 (1954) 121.
- [10] H.K. Chenault and G.M. Whitesides, *Appl. Biochem. Biotech.* 14 (1987) 147.
- [11] S. Pinder and J.B. Clarke, *Methods Enzymol.* 18B (1971) 20.
- [12] M.B. Mathews and E.E. Cohn, *J. Am. Chem. Soc.* 75 (1953) 5428.
- [13] E. Negelein and E. Haas, *Biochem. Z.* 282 (1935) 206.
- [14] P. Greengard, *Nature* 178 (1956) 632.
- [15] B. Birdsall, R.W. King, M.R. Wheeler, C.A. Lewis, S.R. Goode, R.B. Dunlap and G.C.K. Roberts, *Anal. Biochem.* 132 (1983) 353.
- [16] K. Dalziel, *J. Biol. Chem.* 238 (1963) 2850.
- [17] H. Theorell and B. Chance, *Acta. Chem. Scand.* 5 (1951) 1127.