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SPECIFICITY AND OTHER PROPERTIES OF AN ALCOHOL DEHYDROGENASE PURIFIED FROM *COMAMONAS TERRIGENA*

AN ENZYME EXHIBITING PREFERENCE FOR L-STEREOISOMERS OF SECONDARY ALCOHOLS

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An NAD-dependent alcohol dehydrogenase (alcohol : NAD^+ oxidoreductase, EC 1.1.1.1) active towards L-octan-2-ol but not towards the corresponding D-isomer was purified to homogeneity from the soil bacterium *Comamonas terrigena*. The enzyme is a tetramer (molecular weight 125 000–141 000) and is most active at pH 8.5–9.9. Preferred alcohol substrates are L-alkan-2-ols, activity towards which was inhibited by EDTA, 1,10-phenanthroline and 2,2′-bipyridine. The enzyme exhibits much weaker activity towards primary alcohols, symmetrical secondary alcohols and asymmetric secondary alcohols in which the hydroxyl moiety is located at positions other than C-2, and little or no activity towards D-alkan-2-ols. For L-alkan-2-ols, symmetrical secondary alcohols and primary alcohols, log K_m values decrease linearly with increase in the number of carbon atoms in the alkyl chain. A plot of standard free-energy of binding ($\Delta G^{0\prime}$) of substrates against the number of carbon atoms in the alkyl chain (primary alcohols) or the longer of the two portions of the alkyl chain (secondary alcohols) gives a single straight-line relationship, suggesting that hydrophobic interactions make an important contribution to substrate binding. The observed specificity was interpreted in terms of a model in which secondary alcohols interact with the enzyme through the hydrogen and hydroxyl group that participate in NAD^+ reduction, and one of the two alkyl segments. The size of the unbound alkyl segment markedly affects V, the optimum being a single methyl unit. This specificity was correlated with that of the CS2 secondary alkylsulphohydrolase that catalyses the production of L-alkan-2-ols from D-alkan-2-yl sulphate surfactants.

Introduction

Biodegradation of primary and secondary alkyl sulphate surfactants by soil micro-organisms is initiated by removal of the sulphate group via the agency of alkylsulphohydrolases and the liberated alcohol is then oxidized [1–3]. Biodegradation studies in the Cardiff laboratories have focussed on two soil isolates. One of these, *Pseudomonas* C12B, can initiate the degradation of a wide range of primary and secondary alkyl sulphate esters because of its ability to produce up to two primary and three secondary alkylsulphohydrolases, depending on the growth conditions [4]. This bacterium also possesses an NAD-dependent dehydrogenase activity towards primary and secondary alcohols [5]. The other organism, a strain of *Comamonas terrigena*, produces two secondary alkylsulphohydrolases only. One of these (designated CS2) is stereospecific for D-alkan-2-yl sulphates and the other (CS1) is specific for the corresponding L-isomers [6]. Two analogous enzymes (designated as S1 and S2, respectively) are present in *Pseudomonas* C12B [7]. Hydrolysis of alkan-2-yl sulphates by these enzymes is effected by rupture of the C-O bond of the C-O-S linkage, and this is accompanied by inversion of configuration [8].

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C. terrigena also produces two stereospecific NAD-dependent alcohol dehydrogenases. One is active towards D-alkan-2-ols and primary alcohols, and the other oxidizes L-alkan-2-ols, symmetrical alcohols, primary alcohols and certain other positional isomers of secondary alcohols [9]. In comparison with other known alcohol dehydrogenases, the latter enzyme, which will be referred to as L-alkan-2-ol dehydrogenase, exhibits novel specificity. The present paper describes its purification and characterization, and provides further information about this specificity.

Materials

Resolved stereoisomers of butan-2-ol and octan-2-ol were obtained from Fluka AG, Buchs SG, Switzerland and the Aldrich Chemical Co., Milwaukee, WI, U.S.A., respectively. Other straight-chain monohydroxy alcohols were purchased from the Aldrich Chemical Co., Fluka AG, K and K Laboratories, Plainview, NY, U.S.A., Koch Light Laboratories, Colnbrook, Bucks, U.K. or Eastman Organic Chemicals, Rochester, NY, U.S.A. Tridecan-7-ol was a gift from Dr. A.H. Olavesen of this department. Resolved stereoisomers of alkan-2-ols not available commercially were prepared as described by Barrett et al. [10]. Optical isomers of octan-2-yl sulphate were prepared as described by White et al. [11]. Dithiothreitol and pyrazole were supplied by Aldrich Chemical Co., and coenzymes and protein molecular weight standards by Boehringer Mannheim GmbH, F.R.G. Octan-2-one, 2-buten-1-ol and 2,3-butanediol were obtained from Fluka AG. Batyl alcohol, geraniol, farnesol, 1,3,5(10)-estratrien-3,16α,17β-triol and 4-pregnen-17α,21-diol-3,11,20-trione were supplied by Koch Light Laboratories, and 2-(2,4-dichlorophenoxy) ethanol and protease inhibitors were gifts from Mrs. V. Lillis and Dr. J. Kay respectively, both of this department. DEAE-Cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.), Sephadex G-200 and Blue Sepharose CL-6B (Pharmacia, Uppsala, Sweden), and butyl-agarose (Miles Laboratories, Stoke Poges, Bucks, U.K.) were washed and equilibrated before use in accordance with the manufacturers' instructions. All other materials were the purest available from BDH Chemicals or Sigma (London) Chemical Co., both of Poole, Dorset, U.K.

Growth of C. terrigena

The bacterium was grown at 30°C in 350 l nutrient broth in a 400 l-capacity New Brunswick Fermentor aerated with 1.8 m^3 air/h and agitated at 100 rev./min. Harvesting of cells by Sharples centrifuge (flow-rate 90 l/h) began in late exponential phase of growth and yielded 450 g wet cells. Approx. 50 ml 20 mM Tris-HCl buffer, pH 7.5, were stirred into the cell mass which was stored at −20°C.

Assay and kinetic analysis of alcohol dehydrogenase activity

Routinely, alcohol dehydrogenase activities towards L-octan-2-ol, D-octan-2-ol and octan-1-ol were determined as described previously [9]. The activity of the purified enzyme was proportional to its concentration up to 1.05 μg/assay mixture. Initial reaction rates could not be measured with higher concentrations. For kinetic and specificity studies with pure enzyme, the same method was used except that the type and concentration of the alcohol were varied as required. In each case, the amount of enzyme present was adjusted to produce a convenient rate of reaction. A unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol NADH/min under standard assay conditions. In some cases, kinetic experiments were restricted by the solubility limits of certain alcohols. However, at concentrations below the solubility limits, Michaelis-Menten kinetics were observed. Values of K_m and V obtained from double-reciprocal plots of these data were not significantly different from those obtained by a weighted linear regression analysis.

Purification of L-alkan-2-ol dehydrogenase

All purification procedures were carried out at 0–4°C and enzyme samples were stored at 4°C. Centrifugations were performed at $38\,000 \times g_{av}$ in a 16 × 15 ml fixed-angle rotor of an MSE High Speed 18 centrifuge. Nucleic acid and protein were assayed as described previously [12]. To assess the progress of the purification polyacrylamide gel electrophoresis was performed according to the method of Payne and Painter [13] using the pre-electrophoresis and staining modifications introduced by Barrett et al. [9], and Coomassie brillant blue G-250 for staining protein bands [14].

Stage 1. Frozen cells (100 g) were thawed and

resuspended in 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5, to give a final volume of 200 ml. Cells were ruptured by three passages through a chilled French pressure cell operating at 126 MPa. Debris was removed by centrifuging for 60 min.

Stage 2. Nucleic acid was precipitated from the stage 1 supernatant by slow addition of a 7.5% (w/v) solution of streptomycin sulphate (1 : 10 vol. stirred supernatant). The resulting suspension was stirred for 30 min followed by centrifugation for 45 min. The supernatant was then dialysed for 2 h against three changes of buffer (20 mM NaH_2PO_4/Na_2HPO_4, pH 7.5).

Stage 3. The stage 2 material (124 ml) was heated in a stainless steel beaker at 53°C for 30 s with continuous stirring. After rapid cooling, the precipitated protein was removed by centrifugation (15 min) and discarded.

Stage 4. The supernatant from stage 3 (123 ml) was stirred into a thick paste of DEAE-cellulose (120 g wet weight) equilibrated with 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5. After 90 min with occasional stirring, the buffer was removed by filtration. The ion-exchanger was washed with 100 ml fresh buffer, and the filtrate and washings were combined (buffer wash). A solution of NaCl (125 ml, 0.25 M in the same buffer) was stirred into the resin and the whole was left again for 90 min before filtering. The DEAE-cellulose was washed with 25 ml NaCl buffer solution and the filtrate and washings were combined (NaCl wash). The NaCl wash contained L-alkan-2-ol dehydrogenase.

Stage 5. The NaCl wash was concentrated by means of an Immersible-CX Molecular Separator (Millipore; U.K. Ltd., Abbey Road, London, U.K.). Dehydrogenase activity towards D-octan-2-ol and octan-1-ol precipitated when the volume of the solution had been reduced from 139 to 10 ml, whilst L-alkan-2-ol dehydrogenase remained in solution. Precipitated protein was removed by centrifugation for 15 min, and the supernatant was dialysed overnight against three changes of 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5, containing 1 mM dithiothreitol.

Stage 6. The dialysed enzyme preparation was applied to a column of Blue Sepharose CL-6B (12 × 1.2 cm, equilibrated with dialysis buffer) which was then washed with buffer until the eluate contained no protein (approx. 30 ml). Enzyme was eluted with 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5 containing 1 mM NAD^+. Fractions (2 ml) were collected automatically, assayed for protein and enzyme activity, and those containing L-alkan-2-ol dehydrogenase were combined and stored overnight at 4°C.

Stage 7. Stage 6 material was applied to a column (5.5 × 0.5 cm) of butyl-agarose equilibrated with 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5. The column was washed with 10 ml buffer to remove unwanted protein, followed by a solution of 0.2 M NaCl in buffer to elute the dehydrogenase activity. Fractions (1 ml) were collected automatically and assayed for protein (A_{280}) and enzyme. Active fractions were pooled and stored at 4°C.

Determination of molecular weight

The molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200 (fine grade) equilibrated with 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5. The column was calibrated using $K_2Cr_2O_7$, cytochrome *c*, ovalbumin, bovine serum albumin, yeast alcohol dehydrogenase, β-galactosidase and Blue Dextran. Polyacrylamide gel electrophoresis with two different concentrations of Cyanogum 41 [15] was also used to determine the molecular weight of the intact enzyme. Ovalbumin, bovine serum albumin, lactate dehydrogenase, yeast alcohol dehydrogenase and catalase were employed as standards.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [16]. Lysozyme, chymotrypsin, yeast alcohol dehydrogenase, ovalbumin, catalase, bovine serum albumin and β-galactosidase were used as standards.

Results

Enzyme purification

The purification sequence and results are summarized in Table I. Difficulties were encountered during the development of the procedure as each of the alcohol dehydrogenase activities monitored proved to be unstable. Poor recoveries (sometimes total losses) of activity resulted during preliminary investigations of various standard separation techniques, and addition of thiol-protecting reagents

TABLE I

PURIFICATION OF THE L-ALKAN-2-OL DEHYDROGENASE FROM *C. TERRIGENA*

See text for definition of unit of activity and for experimental details.

Purification stage	Protein (mg)	Dehydrogenase activity towards						Specific activity of L-alkan-2-ol dehydrogenase (units/mg)	Purification
		L-Octan-2-ol		D-Octan-2-ol		Octan-1-ol			
		(units)	(% yield)	(units)	(% yield)	(units)	(% yield)		
1. Cell-free extract	13 000	534	100	471	100	1 140	100	0.041	1
2. Streptomycin sulphate	10 000	527	99	461	98	1 020	90	0.052	1.3
3. Heat treatment	5 530	536	100	435	92	732	64	0.097	2.4
4. DEAE-cellulose									
Buffer wash	3 680	0	–	0	–	145	20	–	–
NaCl wash	538	363	68	255	59	237	32	0.674	16.4
5. Concentration	225	282	53	0	0	0	0	1.25	30.6
6. Blue Sepharose	3.44	180	34	–	–	–	–	52.3	1 280
7. Butyl-agarose	1.10	142	27	–	–	–	–	129	3 140

glycerol, NAD^+, DL-octan-2-ol, $ZnCl_2$ or protease inhibitors, or alteration of the pH of extracts did not alleviate the problem. Total loss of activity once occurred in a frozen solution. In addition the alcohol dehydrogenases were adsorbed onto dialysis membrane, although not on glass or plastic surfaces. As a consequence of these observations the purification procedure was developed so as to eliminate lengthy chromatographic steps and dialysis whenever possible, and all enzyme-containing solutions were stored at 4°C. In spite of their general instability the alcohol dehydrogenases were remarkably stable to heat treatment and this provided a useful purification.

Batch treatment with DEAE-cellulose separated a dehydrogenase active towards octan-1-ol but not towards D- or L-octan-2-ol from the D- and L-alkan-2-ol dehydrogenases (Table I). This primary alcohol dehydrogenase, which was less mobile on polyacrylamide gel electrophoresis than the other two enzymes, had very occasionally been observed in earlier experiments with crude cell extracts. It appeared to be the least stable of the three enzymes. Concentration of the enzyme-containing solution after DEAE-cellulose treatment proved to be an effective way of eliminating one of the two stereospecific dehydrogenases. Activity towards D-octan-2-ol could not be detected in the precipitated protein or in the supernatant solution.

Use of Blue Sepharose CL-6B resulted in a good purification of L-alkan-2-ol dehydrogenase. The enzyme bound to the affinity ligand at pH 6.5 and was eluted in the presence of NAD^+ at pH 7.5. Interestingly, although the enzyme would bind to Blue Sepharose CL-6B in the absence of NAD^+ at pH 6.5, no such interaction occurred at pH 7.5 either in the presence or absence of cofactor. The enzyme is normally extremely unstable at pH 6.5 but the inclusion of dithiothreitol in all buffers of this pH kept losses to a minimum. Once bound on to the affinity column the enzyme could not be eluted with buffer at pH 7.5 in the absence of NAD^+. NAD^+ was also an effective eluting agent at pH 6.5, but the higher pH was chosen because of the instability problem. Polyacrylamide gel electrophoresis revealed that the enzyme eluted from Blue Sepharose CL-6B contained a contaminating protein. However, during the final purification step this protein, as well as NAD^+, was not retained by the butyl-agarose column and these components were absent from the final preparation.

An enzyme purification of 3 140-fold was achieved with a recovery of 26.7% (Table I). On polyacrylamide gel electrophoresis (loadings of 5 and 20 μg) the preparation moved as a single protein band that coincided with the band of dehydrogenase activity towards L-octan-2-ol. Enzyme stability was monitored during storage at 4°C and more than 60% of the activity was lost in 4 weeks. None of the stabilizing agents tested earlier (or bovine serum albumin) was effective in preventing this loss. Storage of pure enzyme at −12°C produced an even greater decrease in activity.

Molecular weight determinations

The molecular weight of the L-alkan-2-ol dehydrogenase was 141 000 and 125 000 as determined by gel filtration and polyacrylamide gel electrophoresis under non-denaturing conditions, respectively. SDS-polyacrylamide gel electrophoresis gave one band corresponding to a molecular weight of 33 500, indicating the native enzyme to be a tetramer (cf. yeast alcohol dehydrogenase, [17–19]).

Optimum pH for enzyme activity

Pure enzyme was assayed in the presence of L-octan-2-ol (6 μmol/ml), 1 mM NAD^+ and buffers of various pH values. Buffers (50 mM) were of the following composition and pH: NaH_2PO_4/Na_2HPO_4, pH 6.3–7.4; Tris-HCl, pH 7.3–8.8; glycine-NaOH, pH 8.6–9.9.

Enzyme activity increased as pH was increased from 6.3 to 8.5 and then remained constant up to pH 9.9. The pH/activity profile was similar to that observed for the enzyme present in crude cell extracts, except that in the latter case activity remained constant above pH 7.5 [9].

Specificity of L-alkan-2-ol dehydrogenase

In the presence of 1 mM NAD^+ the enzyme oxidized homologues of L-alkan-2-ols, symmetrical secondary alcohols and primary alcohols (Table II). At low alcohol concentrations, enzyme activity followed the usual hyperbolic dependence on substrate concentration. However, for some of the less soluble alcohols, the increase in velocity ceased abruptly as

TABLE II

SPECIFICITY OF L-ALKAN-2-OL DEHYDROGENASE FOR ALCOHOL SUBSTRATES

V expressed as μmol NADH produced/min per unit enzyme; concentration of NAD^+ was 1 mM. See text for details.

Alcohol	Concentration range of alcohol used in assay (μmol/ml)	Apparent K_m (mM)	V
L-Alkan-2-ols			
L-Butan-2-ol	0–100	8.33	0.96
L-Octan-2-ol	0– 60	0.79	1.11
L-Nonan-2-ol	0– 50	0.06	0.95
L-Decan-2-ol	0– 20	0.029	0.74
Symmetrical alcohols			
Propan-2-ol	0– 40	15.40	0.53
Pentan-3-ol	0–100	6.85	0.189
Heptan-4-ol	0– 25	4.35	0.093
Nonan-5-ol	0– 10	0.95	0.081
Undecan-6-ol	0– 1.5	0.44	0.031
Tridecan-7-ol	0– 1	0.16	0.019
Primary alcohols			
Ethanol	0– 30	12.35	0.030
Butan-1-ol	0– 10	3.50	0.053
Hexan-1-ol	0– 5	0.72	0.118
Heptan-1-ol	0– 4	0.41	0.145
Octan-1-ol	0– 5	0.083	0.102
Nonan-1-ol	0– 1	0.050	0.071
D-Alkan-2-ols			
D-Butan-2-ol	0– 50	19.6	0.010

the amount of alcohol present approached its solubility limit. That the effect was the result of saturation of the solution with alcohol rather than saturation of the enzyme with substrate was shown by double-reciprocal plots which when extrapolated gave estimates of V that were higher than the maximum observed in practice. For each homologous series of alcohols the apparent K_m (Table II) decreased as the carbon-chain length of the alcohol increased. Values of V for L-alkan-2-ols were reasonably constant and much greater than those for symmetrical or primary alcohols. With primary alcohol substrates V was highest for the C_6 and C_7 alcohols, whereas for the symmetrical alcohols, V declined with increasing chain length.

No enzyme activity was detected towards L-tetradecan-2-ol (1 μmol/ml), decan-1-ol (1 μmol/ml) or methanol (50 mM) but in the first two cases this may reflect the great insolubility of the alcohols under the experimental conditions employed. D-Butan-2-ol was oxidized but at a very low rate (approx. one-hundredth of the rate observed with the L-isomer under similar conditions). Nevertheless, by raising the enzyme and the substrate concentration it was possible to produce measurable rates suitable for estimating K_m and V (Table II). D-Heptan-2-ol was a poorer substrate than D-butan-2-ol and, moreover, because it is much less soluble than the C_4 homologue, it was not possible to obtain the range of substrate concentration necessary for the determination of K_m and V.

Enzyme activity was measured towards various concentrations of NAD^+ (0–1 mM) in the presence of either L-octan-2-ol (6 μmol/ml) or L-butan-2-ol (60 mM). In both cases simple Michaelis-Menten kinetics were observed, and a K_m value for NAD^+ of 0.061 mM was obtained (cf. 0.052 mM obtained with crude cell extracts [9]). $NADP^+$ could not replace NAD^+ as coenzyme.

Determination of kinetic parameters for L-*alkan-2-ols as components of racemic mixtures*

Because D-alkan-2-ols did not inhibit L-alkan-2-ol dehydrogenase (or showed only a very limited ability to inhibit, see later) it was concluded that racemic samples of alkan-2-ols could be used for studies on the kinetic behaviour of the enzyme towards the L-isomer in those cases where resolved enantiomorphs were not available. It was assumed that the two stereoisomers were present in equal amounts for each racemic alkan-2-ol employed. Enzyme activity in the presence of 1 mM-NAD^+ was measured towards DL-butan-2-ol (0–30 mM), DL-pentan-2-ol (0–20 mM), DL-hexan-2-ol (0–10 mM), DL-heptan-2-ol (0–6 mM) and DL-octan-2-ol (0–4 mM), the first and last of these compounds serving as controls because they were also available in resolved forms.

For each racemic alcohol an enzyme activity/substrate concentration curve was constructed after halving the values of the alcohol concentrations that were actually used in the assays. All the curves were hyperbolic, and the appropriate K_m and V values are presented in Table III. Values obtained for L-butan-2-ol and L-octan-2-ol as components of racemic mixtures agreed well with those obtained for each pure isomer (Table II). For the C_4 to C_8 homologous series, the apparent K_m value decreased as the chain length of the alcohol increased, whilst V values again showed little variation.

In general, solubility problems hindered an examination of higher alcohols but DL-dodecan-2-ol (1 μmol/ml) in the presence of 1 mM NAD^+ was shown to react at a very slow rate.

TABLE III

APPARENT K_m AND V VALUES FOR L-ALKAN-2-OL COMPONENTS OF RACEMIC SAMPLES OF ALCOHOL

See text for details; V expressed as μmol of NADH produced/min per unit enzyme.

DL-Alkan-2-ol	Apparent K_m (mM)	V
DL-Butan-2-ol	7.55	0.90
DL-Pentan-2-ol	2.94	0.97
DL-Hexan-2-ol	1.64	1.03
DL-Heptan-2-ol	1.10	1.08
DL-Octan-2-ol	0.61	1.14

Enzyme activity towards various other racemic alcohols

In order to obtain further information on the breadth of the specificity of the enzyme, the rates of oxidation of a number of other alcohols were compared. First, several simple alkanols were examined. Because resolved isomers of alkan-3-ols and alkan-4-ols were unavailable, racemic mixtures were used (Table IV) and DL-alkan-2-ols for which kinetic data were already available were also included for comparison. With the exception of DL-hexan-2-ol and DL-hexan-3-ol each alcohol was present in sufficient quantity to saturate the solution. All the alcohols tested could serve as substrates but DL-alkan-2-ols were again oxidized at much the fastest rates. Second, several rather more complex alcohols were tested as potential substrates with each being present either as a saturated solution or at a concentration of 10 mM, whichever was the higher. None of the 30 compounds tested was a good substrate. Certain primary and secondary alcohols that contained an amino group, a phenyl group, a double bond, additional hydroxy groups or a branched alkyl chain were oxidized but at a low rate (Table IV). The following compounds that might be considered to be potential substrates were not oxidized at all: 2-(2,4-dichlorophenoxy)ethanol; cyclohexanol; 2-methylbutan-2-ol; DL-3-octadecyloxy-1,2-propanediol (batyl alcohol); glycerol; tris(hydroxymethyl) methylamine; DL-lactate; DL-malate; ethanediol; diethylene glycol; triethyleneglycol; poly(ethylene glycol) 200; geraniol; farnesol; cholesterol; androsterone; epiandrosterone; testosterone; epitestosterone; deoxycorticosterone; 20α-hydroxy-Δ^4-pregnen-3-one; 20β-hydroxy-Δ^4-pregnen-3-one; 4-pregnen-17α, 21-diol-3,11,20-trione; 1,3,5(10)-estratrien-3,16α,17β-triol.

Reaction progress curves for the oxidation of positional isomers of secondary octanol

The progress of the oxidation of L-octan-2-ol, DL-octan-2-ol, DL-octan-3-ol and DL-octan-4-ol was monitored. The amount of enzyme present in each assay mixture was 0.10, 0.10 and 1.0 and 1.0 units, respectively. The progress curves indicate that oxidation of L-octan-2-ol (Fig. 1a) proceeded to 92% of the theoretical maximum value and remained unchanged thereafter, whereas, as expected, only 50% of DL-octan-2-ol had reacted under these conditions

TABLE IV

ACTIVITY OF L-ALKAN-2-OL DEHYDROGENASE TOWARDS RACEMIC MIXTURES OF SECONDARY ALCOHOLS

Concentration of NAD^+ was 1 mM. See text for details.

Alcohol	Concentration in assay (μmol/ml)	Rate of oxidation (μmol of NADH produced/min per unit enzyme)
Simple alkanols		
DL-Hexan-2-ol	70	0.98
DL-Heptan-2-ol	70	1.04
DL-Octan-2-ol	7	1.00
DL-Nonan-2-ol	4	0.93
DL-Hexan-3-ol	70	0.31
DL-Heptan-3-ol	70	0.24
DL-Octan-3-ol	10	0.14
DL-Nonan-3-ol	4	0.11
DL-Decan-3-ol	2	0.05
DL-Undecan-3-ol	1	0.03
DL-Octan-4-ol	10	0.15
DL-Nonan-4-ol	4	0.12
DL-Decan-4-ol	2	0.06
DL-Decan-5-ol	2	0.07
Other alcohols		
DL-1-Aminopropan-2-ol	10	0.050
2,3-Butanediol	10	0.066
L-α-Hydroxyphenylacetic acid	10	0.045
3-Phenyl-2-propan-1-ol	1	0.054
2-Buten-1-ol	10	0.016
2-Ethylhexan-1-ol	2	0.009

(Fig. 1b). DL-Octan-3-ol was oxidized at a much slower rate than the corresponding C-2 isomer, despite a 10-fold higher concentration of enzyme. Although this rate declined rapidly, the reaction did not stop at the 50% stage but progressed steadily to reach 69% oxidation after 100 min (Fig. 1c). For DL-octan-4-ol the rate of reaction was initially even slower than for DL-octan-3-ol but the deceleration was less marked so that the oxidation had advanced to 85% in 100 min (Fig. 1d). Both stereoisomers of octan-3-ol and octan-4-ol can therefore serve as substrates for L-alkan-2-ol dehydrogenase. However, with DL-octan-3-ol one enantiomer, presumably the L-isomer, is clearly a better substrate. The ability of the enzyme to discriminate between optical isomers is less apparent for octan-4-ol.

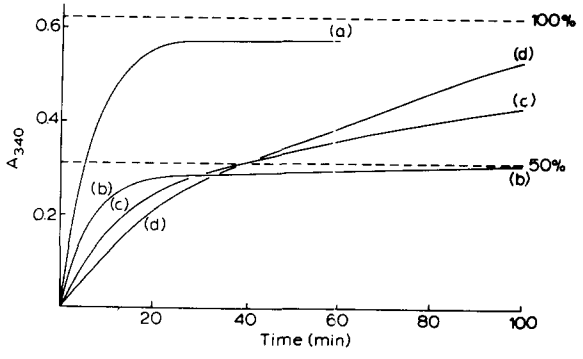

Fig. 1. Reaction progress curves for the oxidation of asymmetric secondary octanols catalysed by L-alkan-2-ol dehydrogenase. Incubation mixtures (1.4 ml) contained 5 mM NAD^+, 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 8.5 and 0.1 mM alcohol. (a) L-Octan-2-ol; (b) DL-octan-2-ol; (c) DL-octan-3-ol; (d) DL-octan-4-ol. The amount of enzyme present was 0.1 unit for (a) and (b), and 1.0 unit for (c) and (d).

Inhibition of L-*alkan-2-ol dehydrogenase*

The ability of a variety of compounds to inhibit enzyme activity towards 2 mM L-octan-2-ol was

investigated in the presence of 1 mM NAD^+. None of the following compounds was inhibitory under the conditions used; D-octan-2-ol (4.6 μmol/ml); D-nonan-2-ol (2.3 μmol/ml); D-decan-2-ol (1.1 μmol/ml); D-dodecan-2-ol (0.6 μmol/ml); D-tetradecan-2-ol (0.3 μmol/ml); D-octan-2-yl sulphate (10 mM); L-octan-2-yl sulphate (10 mM); pyrazole (10 mM). All alcohols other than the substrate were present in assay mixtures at concentrations exceeding their maximum solubilities. D-Butan-2-ol (22.9 mM) and D-heptan-2-ol (11.4 mM) were present at concentrations less than their maximum solubilities and were feeble inhibitors (20 and 10% inhibition, respectively).

The effect of chelating agents on enzyme activity was also studied. Samples of suitably diluted enzyme containing either EDTA, 1,10-phenanthroline or 2,2′-bipyridine (100 μM each) were incubated at room temperature for 30 min prior to assay by the standard procedure. Controls contained no chelating agent. L-Alkan-2-ol dehydrogenase activity was inhibited 16% by EDTA and 50% by either 1,10-phenanthroline or 2,2′-bipyridine, implying that enzyme activity requires the presence of a metal ion.

Discussion

The scheme employed enabled L-alkan-2-ol dehydrogenase to be purified 3 140-fold from crude cell extracts. The magnitude of this purification factor is of the same order as that achieved (4 290-fold) for the constitutive D-secondary alkylsulphohydrolase (CS2) of this bacterium [6], the enzyme that would hydrolyse D-alkan-2-yl sulphates to yield the L-alkan-2-ol substrates of the dehydrogenase. In terms of cell protein, the two enzymes are present in the cell in roughly equal amounts.

The pure preparation of L-alkan-2-ol dehydrogenase was produced in reasonable yield in spite of the general instability of the enzyme. The problem of instability has been encountered with other sources of alcohol dehydrogenase activity, for example, rat liver [20] and *Pseudomonas aeruginosa* [21,22]. Although thiol reagents and other substances have been included as protective agents during other purification procedures they were not effective with the L-alkan-2-ol dehydrogenase.

In an earlier study [9], two alcohol dehydrogenases were always detected on gel electrophoretograms of crude cell extracts of *C. terrigena*. The faster moving band was the L-alkan-2-ol dehydrogenase and the slower band was active towards both primary (octan-1-ol) and secondary (D-octan-2-ol) alcohols. In several different crude extracts that were examined at the time, the ratio of activities exhibited towards octan-1-ol and D-octan-2-ol was generally fairly constant at about 1.2 : 1. However, on two occasions the ratio was 1.7 : 1 and, in the present purification studies, had increased to 2.4 : 1 in the crude extracts of cells grown on a large scale. Gel electrophoretograms of the latter extracts showed a third band of enzyme activity, specific for primary alcohols. This enzyme is the unstable primary alcohol dehydrogenase that was eliminated at stage 4 of the purification procedure. Following its removal, the ratio of activities of the preparation at that stage fell to 0.9 : 1. It therefore seems probable that the ratio variations observed reflect (at least in part) the instability of the primary alcohol dehydrogenase.

L-Alkan-2-ol dehydrogenase is an NAD-dependent enzyme active towards primary and secondary alcohols but with a marked specificity for L-alkan-2-ols. This appears to be the first time that such a preferred stereospecificity has been noted for this class of enzyme. Yeast alcohol dehydrogenase oxidizes primary alcohols and is active towards D-alkan-2-ols but no other secondary alcohols [23]. Horse liver alcohol dehydrogenase is a primary enzyme which also oxidizes secondary alcohols but with a preference for D-isomers [23,24]. Therefore, L-alkan-2-ol dehydrogenase is of an analogous but opposite stereospecificity to the horse liver enzyme. However, the latter is active towards cyclohexanol and 3β-hydroxy-steroids [25,26], whereas L-alkan-2-ol dehydrogenase is not.

Inhibition of enzyme activity by metal chelators, especially 1,10-phenanthroline and 2,2′-bipyridine, was observed for L-alkan-2-ol dehydrogenase and has been reported for several other alcohol dehydrogenases. Enzymes from human and horse livers, and yeast are all inhibited by such chelating agents [27–29]. For the latter two dehydrogenases 1,10-phenanthroline interacts with enzyme-bound zinc, the metal ion being necessary for enzyme activity [30]. Inhibition of L-alkan-2-ol dehydrogenase by 1,10-phenanthroline may indicate the presence of

zinc in the enzyme but this remains to be confirmed. Yeast and liver alcohol dehydrogenases are competitively inhibited by pyrazole but the inhibitor is much less effective towards the former enzyme [31]. However, no inhibition of L-alkan-2-ol dehydrogenase activity by pyrazole was observed. The different effects of this compound presumably reflect differences in the active sites of the dehydrogenases.

An inverse relationship between apparent K_m and alkyl chain lengths of substrates has been observed for many alcohol dehydrogenases including the enzyme from human liver [27], the NAD-dependent soluble enzyme of *Candida tropicalis* [32], an NAD(P)-independent primary alcohol dehydrogenase from a *Pseudomonas* species [33] and NADP-dependent enzymes of *Ps. aeruginosa* [21,34]. For all these activities and for the yeast and horse liver enzymes [35] hydrophobic interactions were considered to play an important role in substrate binding. Kinetic data presented in Tables II and III show for each homologous series of alcohol substrates of L-alkan-2-ol dehydrogenase, the apparent K_m values decrease as the number of carbon atoms in the alcohol increases, again indicating an important contribution from hydrophobic interactions. Because each K_m was less than the solubility of the corresponding alcohol, the trends apparent in Tables II and III are independent of solubility differences. Preliminary examination (Barrett, C.H., unpublished data) of steady-state kinetics and product inhibition patterns using L-octan-2-ol as substrate, suggested a random order rapid equilibrium mechanism for the enzyme. If it is assumed that this mechanism operates for all alcohols, the K_m values for the various alcohols may be interpreted as dissociation constants and values of $\Delta G^{0\prime}$ derived from them serve as measures of the free-energy of binding of each substrate to the enzyme. The relationships between $\Delta G^{0\prime}$ and the number of carbon atoms in the alkyl chain for L-alkan-2-ols, symmetrical secondary alcohols and primary alcohols are shown in Fig. 2a. For each homologous series of substrates a straight-line relationship exists between carbon-chain length and standard free-energy of binding. The increments for binding each successive $-CH_2-$ group are −2.21, −1.72, −1.17 and −2.03 kJ/mol for pure L-alkan-2-ols, L-alkan-2-ols as components of racemic mixtures, symmetrical secondary alcohols and primary alcohols, respectively. The value of the increment for symmetrical alcohols is approx. half that for L-alkan-2-ols and this particular observation suggests that an alternative presentation of the data may be informative.

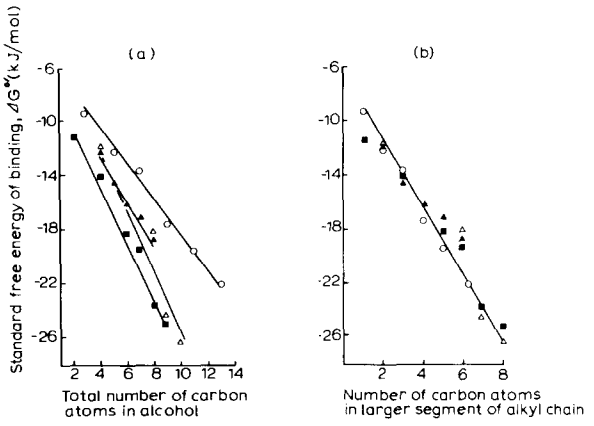

Fig. 2. Linear free-energy relationships for binding various homologous series of alcohols to L-alkan-2-ol dehydrogenase. (a) Free-energy of binding as a function of total number of carbon atoms in each alcohol; (b) free-energy of binding as a function of the number of carbon atoms in the larger alkyl segment. ▲, L-Alkan-2-ols (resolved isomers); △, L-alkan-2-ols (as components of racemic mixtures); ○, symmetrical secondary alcohols; ■, primary alcohols.

The fact that two $-CH_2-$ units must be added to a symmetrical alcohol to achieve the same free-energy increment as the addition of one unit to an L-alkan-2-ol or primary alcohol suggests that only one of the two alkyl segments of a secondary alcohol is involved in hydrophobic binding. If this is the case then the free-energy of binding of an alcohol should be related not to the total number of carbon atoms in the molecule but to the number of carbon atoms in the larger segment of the alkyl chain, discounting the carbinol carbon atom (e.g., C_6 for L-octan-2-ol, C_3 for heptan-4-ol and C_5 for hexan-1-ol). When the data in Fig. 2a are re-plotted in this way, a single straight-line relationship is obtained (Fig. 2b) embracing all the data for various types of alcohol.

The free-energy increment for binding each successive methylene unit now emerges as −2.35 kJ/mol, indicating a high sensitivity to hydrophobic interactions. The linear relationship extends over a range of alkyl segments from C_1 to at least C_8, implying that each of the carbons in the longer alkyl segment contributes to hydrophobic binding. It was not possible

to establish an upper limit to the size of the hydrophobic binding site because of solubility problems encountered with higher alcohols, although DL-dodecan-2-ol did serve as a poor substrate. The occurrence of a hydrophobic binding site immediately adjacent to the site of oxidation contrasts with the situation in the soluble primary alcohol dehydrogenase of *Candida torulopsis* in which the alkyl group binding site is far removed from the active centre so that only long-chain alcohols are substrates [32].

A prerequisite for enzyme stereospecificity is a three-point interaction between enzyme and substrate, as postulated for the CS2 and S3 alkylsulphohydrolases [6,36]. For L-alkan-2-ol dehydrogenase, one of the substrate groups involved in the interaction is obviously the longer of the two portions of the alkyl chain of L-alkan-2-ols. From the foregoing it appears that the C-1 methyl group of L-alkan-2-ols, and one of the two segments of the alkyl chain of symmetrical secondary alcohols, do not participate in substrate binding. It follows that the interaction of the enzyme with the hydrogen and hydroxyl group of the carbinol moiety must be important. This is perhaps not surprising as it is with the transfer of hydrogen from these positions that the enzyme is concerned.

The binding of L-octan-2-ol to the dehydrogenase through these three groups is depicted in Fig. 3a. The longer (C_6) segment of the alkyl chain is bound in a hydrophobic binding site and the shorter segment (C_1 methyl group) remains unbound. The hydroxyl group is bound at a specific site which, in the light of the current views on alcohol dehydrogenase mechanisms [37] and the inhibition of L-alkan-2-ol dehydrogenase by chelating agents, probably involves a metal ion. The C-2 hydrogen atom is now correctly orientated for transfer to NAD^+ to occur. In contrast, the failure to detect inhibition by D-octan-2-ol of enzyme activity towards the L-stereoisomer suggests that none of the conformers of the D-stereoisomer shown in Fig. 3b–d can bind to any significant extent to the enzyme. Enzyme-substrate complexes involving a symmetrical secondary alcohol (e.g., nonan-5-ol) and a primary alcohol (e.g., hexan-1-ol) are depicted in Fig. 3e and f. In these cases also, binding involves contributions from the -OH group, the adjacent hydrogen and part of the alkyl chain.

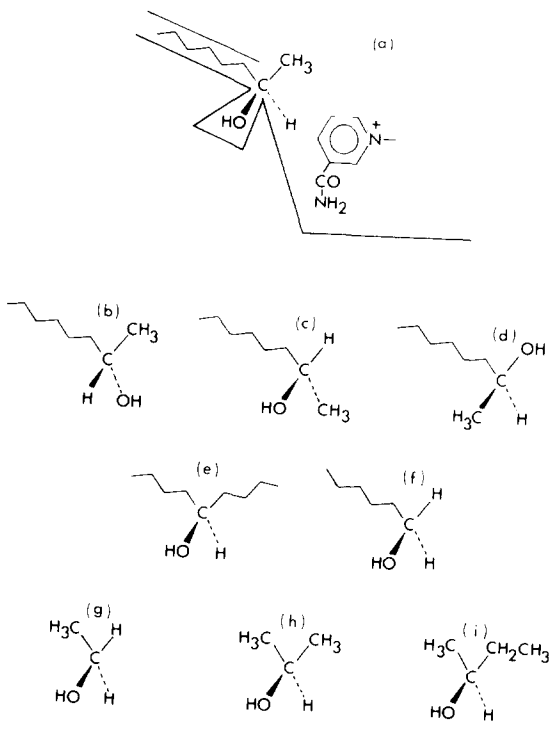

Fig. 3. Proposed model of alcohol binding to L-alkan-2-ol dehydrogenase. (a) Binding of L-octan-2-ol, with longer alkyl chain at hydrophobic binding site, methyl group unbound, hydroxyl group at specific binding site and hydrogen atom correctly orientated with respect to NAD^+ (represented by nicotinamide moiety at coenzyme binding site); (b), (c) and (d) conformers of D-octan-2-ol that cannot bind to the enzyme; (e) and (f) conformations of a symmetrical alcohol substrate and primary alcohol substrate respectively that would bind at the model site; (g), (h) and (i) conformations of ethanol, propan-2-ol and D-butan-2-ol substrates that bind at the model site.

Turning now to the V values obtained for the various substrates another interesting feature emerges. For L- and DL-alkan-2-ols of chain length C_4 to C_9, V values remain remarkably constant (Tables II and III). Clearly the length of the alkyl chain, so important in the binding of substrate to enzyme, is of little significance to the catalytic step. A somewhat similar situation exists for C_6–C_9 primary alcohols although the actual values are generally only about 10–15% of those for the L- and DL-alkan-2-ols. It appears that high enzyme activity is obtained when the unbound substrate group (see Fig. 3a) is $-CH_3$, whereas weak

activity is observed when the unbound moiety is hydrogen (primary alcohols).

V values for symmetrical alcohols, with the exception of propan-2-ol, are again much lower than those for L- and DL-alkan-2-ols and tend to decrease with increasing chain length. These findings support the view that a single 'unbound' methyl group (propan-2-ol) is necessary for high enzyme activity and also indicate that 'unbound' chain segments larger than $-CH_3$ do reduce catalytic efficiency in some way which remains obscure for the moment.

With this enzyme model in mind, it is instructive to consider the ability of D-butan-2-ol to serve as a substrate, albeit a poor one. The K_m of this substrate (19 mM) is very similar to the values obtained for ethanol (12.4 mM) and propan-2-ol (15.4 mM), both of which, according to the model, are attached in the same way to the enzyme with the hydroxyl group and adjacent hydrogen in the appropriate positions and with a methyl group in the hydrophobic site (Fig. 3g and h). D-Butan-2-ol is presumed to bind in the same way (Fig. 3i), the only difference among the three compounds being the nature of the unbound moiety. Under these circumstances it would be expected that the K_m values for the three alcohols would be of the same order. The low V of D-butan-2-ol (0.01) relative to that of propan-2-ol (0.53) presumably reflects the hindrance to the catalytic step incurred by having an unbound segment larger than a single methyl group.

Higher homologues of the series of D-alkan-2-ols should also bind in an analogous way but because large unbound segments lead to low V values, such compounds would not be expected to be good substrates. This was indeed the case for D-heptan-2-ol (activity weaker than D-butan-2-ol) and D-octan-2-ol (no activity). Unfortunately because of considerable difficulties experienced in separating stereoisomers from some racemic mixtures it has not been possible to prepare C_5 and C_6 representatives of a C_4–C_8 series of such isomers. In relation to the failure to detect activity towards D-octan-2-ol it is necessary to ask why a saturated solution of D-octan-2-ol failed to inhibit enzyme activity towards the L-stereoisomer. According to the model the D-isomer should bind to the enzyme with a single methyl group in the alkyl chain binding site. However, the expected K_m for such binding (circa 19 mM D-butan-2-ol) is greatly in excess of the solubility of D-octan-2-ol and it seems certain that the failure to detect inhibition reflects low solubility coupled to poor binding affinity.

The enzymic oxidation of DL-octan-3-ol and DL-octan-4-ol can now be considered. In both cases the oxidation proceeds in two distinct phases (Fig. 1). In the initial and faster phase of DL-octan-3-ol oxidation the L-component is presumed to be the preferred substrate with the C_5 segment of the alkyl chain bound and the C_2 segment unbound. In the second and slower phase when the residual concentration of the L-isomer is low, the D-isomer competes successfully for the active site but because the positions of the alkyl segments are now reversed, the K_m is higher and V lower than for the L-isomer. Both factors would contribute to a lower oxidation rate. An analogous situation was noted for DL-octan-4-ol (Fig. 1) although the difference between the isomers is less pronounced, the initial and faster phase being slower (binding via a C_4 segment) than that observed for DL-octan-3-ol, and the second phase being faster (binding via a C_3 segment) than that observed for DL-octan-3-ol.

Finally, in a broader context it is interesting to consider the specificity of L-alkan-2-ol dehydrogenase in relation to the degradation of secondary D-alkan-2-yl sulphate esters by *C. terrigena*. The first degradation step is removal of the sulphate ester group by the CS2 secondary alkylsulphohydrolase. This enzyme is absolutely specific for D-alkan-2-yl sulphates [6] and although it will bind a variety of primary and secondary alkyl sulphate esters, only the D-alkan-2-yl sulphates possess the C-1 methyl group in the correct orientation for catalytic hydrolysis to occur [10]. The reaction proceeds with C-O bond cleavage and inversion of configuration at C-2 to produce L-alkan-2-ols that then serve as substrates for the second enzyme in the sequence, the L-alkan-2-ol dehydrogenase. Activity of this enzyme also depends on the presence of the C-1 methyl group, this time in the L-configuration. The sulphohydrolase and dehydrogenase are thus well matched since the L-alkan-2-ol products of the former are the preferred substrates of the latter. Evidently a key factor, both in the ability of these two enzymes to act in sequence and in their overall specificity for degrading D-alkan-2-yl sulphate ester substrates to achiral ketones, is the presence and orientation of the C-1 methyl

group. Although *C. terrigena* can utilize D-octan-2-yl sulphate as energy source [9] it seems improbable that the organism has acquired the sulphohydrolase/dehydrogenase system only since the advent of man-made alkyl sulphate detergents. Presumably the organism has encountered natural secondary alkyl sulphates of some kind since time immemorial. The narrow specificity of the sulphohydrolase/dehydrogenase pathway for D-alkan-2-yl sulphates/L-alkan-2-ols may therefore be an important clue to the identity of the natural alkyl sulphate ester substrates for which the enzymes were originally evolved.

References

1 Williams, J.P. and Payne, W.J. (1964) Appl. Microbiol. 12, 360–362
2 Payne, W.J., Williams, J.P. and Mayberry, W.R. (1967) Nature (London) 214, 623–624
3 Lijmbach, G.W.M. and Brinkhuis, E. (1973) Antonie van Leeuwenhoek 39, 415–423
4 Dodgson, K.S., Fitzgerald, J.W. and Payne, W.J. (1974) Biochem. J. 138, 53–62
5 Williams, J.P., Mayberry, W.R. and Payne, W.J. (1966) Appl. Microbiol. 14, 156–160
6 Matcham, G.W.J., Dodgson, K.S. and Fitzgerald, J.W. (1977) Biochem. J. 167, 723–729
7 Bartholomew, B., Dodgson, K.S. and Gorham, S.D. (1978) Biochem. J. 169, 659–667
8 Bartholomew, B., Dodgson, K.S. Matcham, G.W.J., Shaw, D.J. and White, G.F. (1977) Biochem. J. 165, 575–580
9 Barrett, C.H., Dodgson, K.S., White, G.F. and Payne, W.J. (1980) Biochem. J. 187, 703–709
10 Barrett, C.H., Dodgson, K.S. and White, G.F. (1980) Biochem. J. 191, 467–473
11 White, G.F., Lillis, V. and Shaw, D.J. (1980) Biochem. J. 187, 191–196
12 Cloves, J.M., Dodgson, K.S., White, G.F. and Fitzgerald, J.W. (1980) Biochem. J. 185, 23–31
13 Payne, W.J. and Painter, B.G. (1971) Microbios 3, 199–206
14 Diezel, W., Kopperschläger, G. and Hofmann, E. (1972) Anal. Biochem. 48, 617–620
15 Zwann, J. (1967) Anal. Biochem. 21, 155–168
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4 406–4 412
17 Hersh, R.T. (1962) Biochim. Biophys. Acta 58, 353–354
18 Ohta, T. and Ogura, Y. (1965) J. Biochem. (Tokyo) 58, 73–89
19 Bühner, M. and Sund, H. (1969) Eur. J. Biochem. 11, 73–79
20 Björkhem, I. (1972) Eur. J. Biochem. 30, 441–451
21 Tassin, J.P. and Vandecasteele, J.P. (1972) Biochim. Biophys. Acta 276, 31–42
22 Tassin, J.P., Celier, C. and Vandecasteele, J.P. (1973) Biochim. Biophys. Acta 315, 220–232
23 Dickinson, F.M. and Dalziel, K. (1967) Biochem. J. 104, 165–172
24 Dalziel, K. and Dickinson, F.M. (1966) Biochem. J. 100, 34–36
25 Merrit, A.D. and Tomkins, G.M. (1959) J. Biol. Chem. 234, 2 778–2 782
26 Reynier, M., Theorell, H. and Sjörvall, J. (1969) Acta Chem. Scand. 23, 1 130–1 136
27 Lange, L.G., Sytkowski, A.J. and Vallee, B.L. (1976) Biochemistry 15, 4 687–4 693
28 Vallee, B.L. and Hoch, F.L. (1957) J. Biol. Chem. 225, 185–195
29 Dalziel, K. (1963) Nature (London) 197, 462–464
30 Vallee, B.L., Coombes, T.L. and Williams, R.J.P. (1958) J. Am. Chem. Soc. 80, 397–401
31 Reynier, M. (1969) Acta Chem. Scand. 23, 1 119–1 129
32 Lebeault, J.M., Meyer, F., Roche, B. and Azoulay, E. (1970) Biochim. Biophys. Acta 220, 386–395
33 Van der Linden, A.C. and Huybregtse, R. (1969) Antonie van Leeuwenhoek 35, 344–360
34 Tassin, J.P. and Vandecasteele, J.P. (1971) C.R. Acad. Sci. Paris 272, 1 024–1 027
35 Dickinson, F.M. and Dalziel, K. (1967) Nature (London) 214, 31–33
36 Shaw, D.J., Dodgson, K.S. and White, G.F. (1980) Biochem. J. 187, 181–190
37 Branden, C.-I., Jörnvall, H. and Furugren, B. (1975) in The Enzymes (Boyer, P.D., ed.), Vol. XI, pp. 313–328, Academic Press, New York