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The stereochemistry of hydrogen transfer catalysed by D(−)-3-hydroxybutyrate dehydrogenase

D(−)-3-Hydroxybutyrate dehydrogenase (D(−)-3-hydroxybutyrate–NAD$^+$ oxidoreductase, EC 1.1.1.30) has recently been crystallised from extracts of *Rhodopseudomonas spheroides*[1]. In the present study the stereospecificity of hydrogen transfer from the pyridine nucleotide has been ascertained for the enzyme by the use of reduced coenzyme labelled with tritium at position 4 of the dihydropyridine ring. By comparison with enzymes of known stereospecificity[2] it is concluded that tritium is transferred to acetoacetate from the 4-S derivative ('B' side specific labelling)[3].

[1-^{3}H]Ethanol, specific activity 25.0 mC/mmole and [4-^{3}H]NAD$^+$ ([^{3}H NAD$^+$), specific acitivity 262.0 mC/mmole were obtained from New England Nuclear Corporation, Boston, Mass. Lactate, glutamate, alcohol (horse liver) and 3-hydroxybutyrate dehydrogenases were the products of C.F.Boehringer and Soehne,Mannheim, Germany. Counting was done in Model 3002 Tri-carb Scintillation Spectrometer (Packard Instrument Company) at 60% gain. Sample quenching was determined by the channels ratio method[4].

The experimental procedure is shown in Table I. The results have been expressed

TABLE I

STEREOSPECIFICITY OF 3-HYDROXYBUTYRATE DEHYDROGENASE FOR NAD$^+$

Reaction mixtures were prepared to contain about 2 μmoles of [^{3}H]NAD$^+$ or NAD$^+$, 50 μmoles of Tris buffer (pH 9.4) or phosphate (pH 8.4), excess substrate and 20 μg of enzyme in a volume of 4 ml. In the reoxidation step aliquots of the NADH from the cellulose column were used directly; 20 μg of oxidising enzyme and 0.5–0.6 μmole of substrate being added. Both oxidation and reduction were followed by absorbance changes at 340 mμ. Note that reduction of [^{3}H]NAD$^+$ by an enzyme of A-stereospecificity gives B-NAD^{3}H, whereas reduction of [^{3}H]NAD$^+$ by an enzyme of B-stereospecificity gives A-NAD^{3}H.

Expt. No.	*Reducing enzyme and substrate*	*Oxidising enzyme and substrate*	*Tritium removed (%)*
1	Alcohol dehydrogenase, ethanol, [^{3}H]NAD$^+$, A	Lactate dehydrogenase, pyruvate, A	20
		3-Hydroxybutyrate dehydrogenase, acetoacetate	34
		Glutamate dehydrogenase, α-oxoglutarate, NH_3, B	34
2	3-Hydroxybutyrate dehydrogenase, hydroxybutyrate, [^{3}H]NAD$^+$	Glutamate dehydrogenase, α-oxoglutarate, NH_3,B	9
		Lactate dehydrogenase, pyruvate, A	35
3	Alcohol dehydrogenase, [1-^{3}H]ethanol, NAD$^+$, A	Lactate dehydrogenase, pyruvate, A	98
		Alcohol dehydrogenase, acetaldehyde, A	96
		3-Hydroxybutyrate dehydrogenase, hydroxybutyrate	19
4	Glutamate dehydrogenase, glutamate, [^{3}H]NAD$^+$, B	3-hydroxybutyrate dehydrogenase, hydroxybutyrate	14

numerically as the percentage of tritium removed from the nicotinamide moiety on reoxidation of the reduced pyridine nucleotide although in all cases, except the alcohol produced in Expt. 3, the substrates were isolated by thin-layer chromatography and also counted. Enzyme reactions were stopped by heating for 90 sec. In the reduction step the NAD^+ and NADH were separated on a column of DEAE-cellulose (Whatman DE 32, microgranular) using 0.2 M triethylamine–bicarbonate buffer (pH 7.6). In this system the oxidised and reduced forms were eluted at approx. 1.7–1.9 and 3.7–3.9 column volumes, respectively. In the oxidation step after heating the solution was freeze-dried. The residues were dissolved in a small amount of water containing acetic acid (1 μM) and aliquots were spotted onto thin-layer plates coated with silica G gel (0.3 mm thickness). NAD^+ and the corresponding substrates were added as carriers. The plates were run for 2 h in ethanol–1 M ammonium acetate (70:30, v/v)[5]. NAD^+ was well separated from lactate, 3-hydroxybutyrate and glutamate in this system. It is essential to separate the components of the oxidation mixture by thin-layer chromatography, since NAD^+ and reduced substrates co-chromatographed on the DEAE-cellulose. After drying at room temperature the plates were exposed to iodine vapour and the relevant spots marked. The specific areas of silica gel were scraped into Tri-carb counting vials. The scrapings were suspended in 15 ml of 4% (w/w) Cab-O-Sil toluene scintillation solution containing 5 g 2,5-diphenyloxazole (PPO) and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) per l of toluene[6].

From the results in Table I it is evident that 3-hydroxybutyrate dehydrogenase is a 'B' specific enzyme. That only 34–35% of the tritium could be removed from the coenzyme in Expts. 1 and 2 does not invalidate this conclusion. Additional experiments confirmed that not more than 35% of the tritium could be removed on reduction and reoxidation by other enzyme systems. These results can be explained by the fact that the commercial sample of tritiated NAD^+ must have contained tritium in positions other than at C-4. This was not the case when NAD^3H was prepared enzymatically using [1-^{3}H]ethanol and alcohol dehydrogenase. As is seen in Table I (Expt. 3) 98% of the label was removed on reoxidation.

In all the experiments the results deviate from the theoretical value to a variable extent. In previous experiments[7] in which tritium was used for the determination of stereospecificity of other dehydrogenases for NAD^+, theoretical values were not obtained either. This has been attributed to the fact that reduction and reoxidation are never complete and that there is always a variable, though limited, nonenzymic reoxidation of the reduced pyridine nucleotide on heating.

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Preparation of water-insoluble, enzymatically active derivatives of subtilisin type Novo by cross-linking with glutaraldehyde

A variety of methods has been described for the coupling of proteins to insoluble carriers without loss of biological activity. In some cases the protein itself was made insoluble through a cross-linking reaction by means of a chemical reagent[1]. Glutaraldehyde was used by QUIOCHO AND RICHARDS[2] and by HABEEB[3] to prepare water-insoluble, enzymatically active derivatives of carboxypeptidase and trypsin, respectively. In the present report glutaraldehyde has been used to prepare an insoluble derivative of subtilisin type Novo (EC 3.4.4.16), which retained 10–15% of the specific activity of the untreated subtilisin.

Crystalline subtilisin type Novo, batch ch 31c, was a gift from the Novo Pharmaceutical Company. 50-mg aliquots of this enzyme were dissolved in 1.0 ml of 0.1 M

TABLE I

THE FORMATION OF INSOLUBLE SUBTILISIN TYPE NOVO, BY REACTION WITH GLUTARALDEHYDE UNDER VARIOUS CONDITIONS

Subtilisin type Novo (50 mg) was dissolved in 1 ml acetate buffer (pH 5.0) or phosphate buffer (pH 6.0 to 8.0) and ammonium sulphate or acetone was added as shown. The reaction was initiated by addition of 80 μl of 25% glutaraldehyde at room temperature.

Reaction mixture										
Acetate or phosphate buffer, pH	5.0	5.0	5.0	5.0	6.0	7.0	8.0	5.0	5.0	5.0
Ammonium sulphate, percentage satd.	0	30	40	50	50	50	50	0	0	0
Acetone, ml/ml	0	0	0	0	0	0	0	0.6	0.8	1.0
Time of reaction, min	30	30	30	30	30	30	30	60	60	60
Reaction product										
Percentage yield by weight	0	12.6	32.8	48.6	21.6	63.6	73.2	42.0	48.0	42.0
Percentage yield of enzyme activity	0	1.6	4.7	6.1	2.9	4.6	1.7	4.8	4.1	5.0
Specific activity in percentage of specific activity of native enzyme	0	13.4	14.4	12.5	13.4	7.2	2.3	11.4	8.0	11.8

acetate or phosphate buffer of the desired pH and ammonium sulphate or acetone was added as indicated in Table I. After addition of 0.08 ml of a 25% solution of glutaraldehyde (commercial product from Fluka, A.G.) the reaction mixture was stirred for 30 min or 1 h at room temperature. The precipitate was isolated by centrifugation and