Prokaryotic 20β-hydroxysteroid dehydrogenase is an enzyme of the 'short-chain, non-metalloenzyme' alcohol dehydrogenase type

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The primary structure of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was determined after FPLC purification of a commercial preparation. Peptides obtained from different proteolytic cleavages were purified by reverse phase HPLC. The 255-residue structure deduced was found to be distantly homologous to those of *Drosophila* alcohol dehydrogenase and several other dehydrogenases, establishing that prokaryotic 20β-hydroxysteroid dehydrogenase as a member of the 'short-chain alcohol dehydrogenase family'. With the enzymes characterized, the identity is greatest (31–34%) towards 4 other prokaryotic dehydrogenases, but the family also includes mammalian steroid and prostaglandin dehydrogenases. These enzymes are low in Cys and have a strictly conserved Tyr residue that appears to be important.

Alcohol dehydrogenase; Steroid dehydrogenase; Amino acid sequence; Protein family; Homology; Tyrosine residue

1. INTRODUCTION

A number of dehydrogenases active on OH-groups of steroids and other substrates have been reported. One of these enzyme activities, 'alcohol dehydrogenase', is in nature represented by at least three separate family lineages [1]. Two of these lineages were early distinguished, a 'short-chain, non-metalloenzyme type', and a 'long-chain, often zinc-containing, enzyme type' [2]. The classical liver and yeast alcohol dehydrogenases belong to the latter family, the insect enzyme to the former, and was long ago found to be different [3].

Divergent members of the 'short-chain dehydrogenase type' have been characterized and additional sugar, steroid and other dehydrogenases were added to the family [2,4–7], as well as species variants for the *Drosophila* alcohol dehydrogenase [8]. The enzymes initially characterized were non-mammalian, but recently mammalian proteins have also been found to belong to this group, thus linking a human prostaglandin [9] and a human steroid dehydrogenase [10,11] to the prokaryotic/insect short-chain dehydrogenase type. Furthermore, a less variable segment and a strictly conserved Tyr residue have been defined [9], opening the possibility for mechanistic studies of this enzyme group, for which structure-function relationships have been little studied thus far. Consequently, additional relationships are of renewed interest, and we decided to investigate the structure of prokaryotic 20β-hydroxysteroid dehydrogenase in order to see what relationship, if any, this enzyme might have to the increasing group of short-chain alcohol dehydrogenases. The enzyme is known under several names and has apparently dual enzyme activities [12,13].

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2. MATERIALS AND METHODS

2.1. *Dehydrogenase*

20β-Hydroxysteroid dehydrogenase (17,20β,21-trihydroxysteroid:NAD oxidoreductase, EC 1.1.1.53) from *Streptomyces hydrogenans* is commercially available as a purified preparation (Boehringer, Mannheim, FRG). This was used as starting material, and the protein suspension was dissolved by a 50-fold dilution with 0.01 M Tris-HCl, pH 8.15. The material was submitted to FPLC on Mono-Q (Pharmacia), eluted with a gradient of 0–0.6 M NaCl in 15 mM Tris-HCl, pH 8.15.

2.2. *Structural analysis*

Initially, the pure protein was reduced with dithiothreitol and treated with ^{14}C-iodoacetate as described [8]. However, when radioactivity measurements and amino acid analysis failed to reveal the presence of cysteine residues, the carboxymethylation pretreatment was abandoned and the enzyme was used for analysis without alkylation.

Treatments with trypsin, Lys-specific protease from *Lysobacter*, and *Staphylococcus* Glu-specific protease (all from Boehringer, Mannheim) were inefficient at standard conditions (0.1 M ammonium bicarbonate, pH 8.1, 37°C, 4 h). Therefore, repeated enzyme additions, initial solubilization in 8 M urea and digestion in 1 M urea by dilution just before protease addition, or separate treatments of post-digest supernatants and precipitates were tested. Treatment with trypsin was carried out for 24 h with several protease additions. The digest was separated by reverse phase HPLC. The sample for Lys-specific cleavage was dissolved in 8 M urea, and digested overnight after dilution to 1 M urea. Post-digestional acidification with 20% acetic acid produced a precipitate. The supernatant was submitted to two-step peptide purification by initial

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Sephadex G-50 chromatography and subsequent reverse phase HPLC [14]. The precipitate was dissolved in 0.1% SDS and purified directly by reverse phase HPLC. The sample from the Glu-specific cleavage was given several enzyme additions and finally fractionated by reverse phase HPLC. Enzymic redigestions of large peptides were performed under standard conditions (0.1 M ammonium bicarbonate, 37°C, 4 h) and with direct reverse phase HPLC for peptide purification. CNBr cleavage (0.1 mg/ml) was performed in 70% formic acid at room temperature for 24 h.

Total compositions were determined with a Beckman 121M analyzer after hydrolysis in evacuated tubes with 6 M HCl/0.5% phenol for 24 h at 110°C. Sequence degradations were carried out with Applied Biosystems 470A gas phase and 477A pulsed liquid phase sequencers. Phenylthiohydantoin derivatives were detected by reverse phase HPLC as described [15] or on-line with the Applied Biosystems 120 system.

3. RESULTS

Direct analysis of a 20β-hydroxysteroid dehydrogenase preparation from *Streptomyces hydrogenans* (Boehringer, Mannheim) by sequence degradation and SDS/polyacrylamide gel electrophoresis revealed incomplete purity. Therefore, the protein was purified by an FPLC step on Mono-Q (Fig. 1) to yield a preparation with essentially one major band on SDS-polyacrylamide gel electrophoresis. This material was utilized for structural analysis as shown in Fig. 2. Combined, about 50 peptides were analyzed from which the primary structure of a 255-residue protein chain could be deduced.

Peptides from all parts of the molecule were obtained. For pure peptides, the compositions by acid hydrolysis and from sequence degradations agreed. A reasonable agreement was also found for the entire protein as shown in Table I.

A few complications, largely associated with bad solubility of the peptides, were encountered with several of the digests. This forced prolonged digestions with resulting false cleavages and repeated peptide fractionations with low recoveries. The tryptic peptides were often obtained in several forms, including truncated varieties (not shown in Fig. 2) derived from the extensive digestions required. Similarly, the Glu-protease digest gave non-traditional cleavages. Even the CNBr fragments were recovered in multiple forms, because of two Met-Thr bonds non-stoichiometrically cleaved (positions 184–185 and 189–190). However, as in other dehydrogenases [14,15] these bonds were cleaved upon subsequent pH changes during purification and analysis. Two overlaps were not recovered in pure peptides (K1/K2 and K2/K3) but they are compatible with analysis of impure fragments (M2 and M3, cf. Fig. 2), with expected cleavages from the enzyme specificities, and with the homologies (below). Finally, lack of Cys in the amino acid sequence (Fig. 2) was unexpected since a Cys residue has been postulated close to the steroid binding site [16,17] and a ^{14}C incorporation corresponding to 0.2–1 mol/mol was now initially found upon iodoacetate treatment, but could not

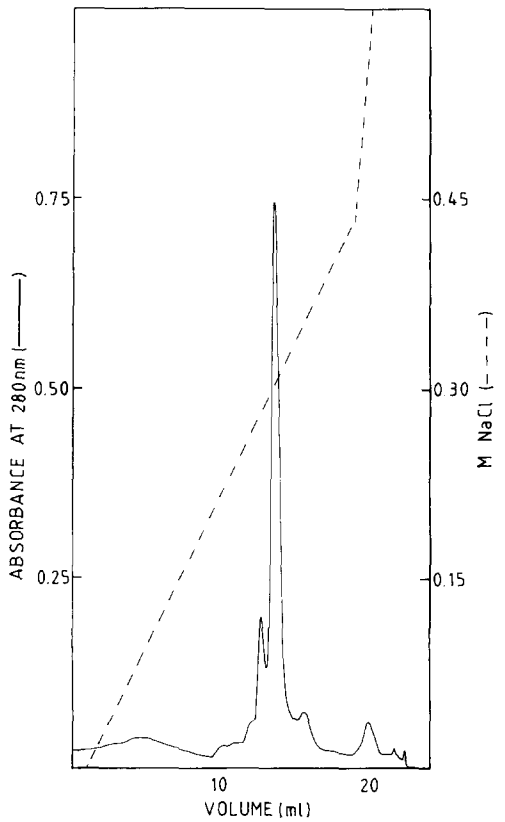

Fig. 1. Purification of 20β-hydroxysteroid dehydrogenase. FPLC on Mono-Q 5/5 in 15 mM Tris-HCl, pH 8.15, with a gradient of 0–0.6 M NaCl. Flow: 1 ml/min. The large peak represents the dehydrogenase.

be detected in the peptides, nor in the preparation analyzed (<0.3 mol/mol). Presumably, the ^{14}C incorporations now first obtained corresponded to low amounts of Cys-containing contaminating protein chains, or to alkylation non-specific for Cys residues. Other reports mentioning Cys [16,17] also seem to lack specific proof.

Combined, the different complications may indicate a reduced reliability at single positions. A few misidentifications should perhaps therefore not be fully excluded. A presence of more than one protein chain, i.e. multiple, structurally closely related enzymes to explain differences, should also not be completely excluded, especially not since the enzyme is reported as bifunctional [12,13], and preparations have varied in both activity (cf. [12]) and composition (cf. Table I and Table in [12]). However, no conclusive evidence for multiplicity exists, and variations appear likely to reflect impurities rather than multiplicities. Significantly, all complications recognized were accounted for, and at the present stage, with the overall compositional agreements (Table I) and entire chain homologies (below), there is no reason for further analysis. The results unambiguously establish that 20β-hydroxysteroid dehydrogenase is a structural relative to other small-chain alcohol dehydrogenases.

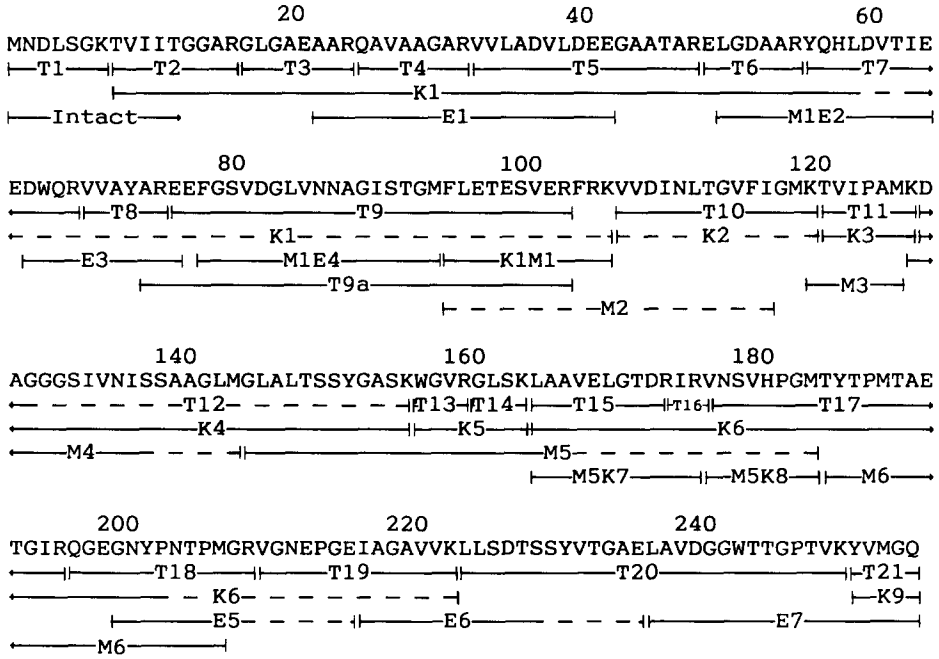

Fig. 2. The primary structure deduced for 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*. Positions of peptides characterized are shown. Solid lines indicate peptide parts analyzed by steps of Edman degradation, dashed lines remaining regions. Peptide names are given by T for trypsin, K for Lys-specific protease, E for Glu-specific protease, and M for CNBr to indicate the cleavages generating each peptide. T9a, M5K7 and M5K8 represent three of the fragments from non-traditional cleavages because of the cleavage conditions necessary (cf. text). Single mis-identifications at a few positions are not fully excluded but compositional data support the assignments (Table I).

Table I

Total composition of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*

Residue	Amount (molar ratios)	
	Acid hydrolysis	Sum of sequence
Cys	0.3	–
Asp	20.3	12
Asn		9
Thr	18.4	20
Ser	14.7	15
Glu	23.6	17
Gln		5
Pro	6.1	7
Gly	35.2	36
Ala	30.6	30
Val	24.5	27
Met	8.1	9
Ile	11.6	12
Leu	19.3	18
Tyr	7.1	7
Phe	5.7	4
Trp	ND	3
Lys	8.1	8
His	2.8	2
Arg	14.4	14
Sum		255

Values as determined after acid hydrolysis (6 M HCl, 24 h, 110°C) and sum of sequence analysis. Hydrolytic value for Cys relates to Cys(Cm) in some preparations after carboxymethylation. ND, not determined.

4. DISCUSSION

The structure of 20β-hydroxysteroid dehydrogenase is clearly but distantly related to those of short-chain alcohol dehydrogenases [2]. An alignment of the enzyme with the first two prokaryotic dehydrogenases of this type characterized [4,18,19] is shown in Fig. 3. Noticeably, these three enzymes represent divergent organisms and completely different enzyme specificities. Residue identities, although not extensive, are clearly discernible (31% between the 20β-hydroxysteroid and glucose dehydrogenases) and cover the entire protein chain (Fig. 3). Consequently, it may be concluded that the 'short-chain dehydrogenase family' includes not only insect alcohol dehydrogenase [2] and prokaryotic dehydrogenases of sugar metabolism [4], but also prokaryotic steroid dehydrogenases. This is compatible with the further inclusion of two other proteins involved in prokaryotic dehydroxylation [5,6] and suggests that they are also hydroxysteroid dehydrogenases. The results enlarge the short-chain alcohol dehydrogenase family and establish it to include many different enzyme specificities. This conclusion fits the recent demonstration that mammalian enzymes are also represented in the group, including some human prostaglandin [9] and steroid [10,11] dehydrogenases. Apparently, this enzyme family is wide-spread and of more general importance than previously known.

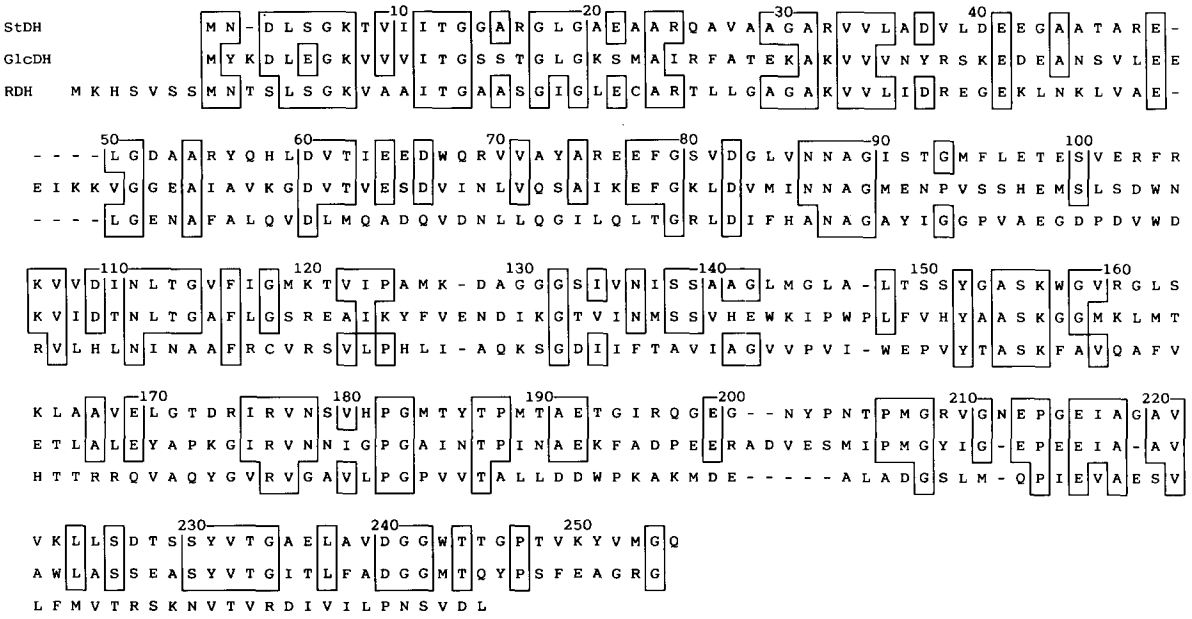

Fig. 3. Alignment of 20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* with the two prokaryotic dehydrogenases initially ascribed to the short-chain alcohol dehydrogenase family. Top line, present analysis with data from Fig. 2. GlcDH, glucose dehydrogenase from *Bacillus megaterium* [18], RDH, ribitol dehydrogenase from *Klebsiella aerogenes* [19]. Residue identities with the present enzyme are boxed. Positional numbers refer to this steroid dehydrogenase but are different in each enzyme because of some gaps (dashes). Alignment of the ribitol dehydrogenase is tentative in the C-terminal segment and there can be given alternative shifts because of low similarity with the other enzymes in this part.

Finally, the homology allows conclusions on functionally important regions. The mechanisms of this group of enzymes have been little studied. Apart from identification of the coenzyme-binding domain to the N-terminal part [20], the presence of a Lys residue in a conserved segment [2], initial observations on reactivities of some residues [18], including His and an acidic residue in 20β-hydroxysteroid dehydrogenase [13,16], and results from site-directed mutagenesis in *Drosophila* alcohol dehydrogenase [21], few data are known about functional aspects. We can now observe that there are large differences in the extent of conservation for separate internal segments of the proteins and that only a few hydrophilic residues of possible functional interest are conserved. No Cys or His is conserved within the three enzymes, but a conserved Tyr residue (Tyr-152), noticed also in relation to prostaglandin dehydrogenase [9] and close to a Lys residue of insect alcohol dehydrogenase [2] (corresponding to Lys-156 in Fig. 3). This segment is of interest for further functional studies, now possible with this and additional enzymes within the family.

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