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ORIGINAL PAPER

Structure and denaturation of 4-chlorobenzoyl coenzyme A dehalogenase from *Arthrobacter* sp. strain TM-1

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Abstract The secondary structure of the trimeric protein 4-chlorobenzoyl coenzyme A dehalogenase from *Arthrobacter* sp. strain TM-1, the second of three enzymes involved in the dechlorination of 4-chlorobenzoate to form 4-hydroxybenzoate, has been examined. E_{mM} for the enzyme was 12.59. Analysis by circular dichroism spectrometry in the far uv indicated that 4-chlorobenzoyl coenzyme A dehalogenase was composed mostly of α-helix (56%) with lesser amounts of random coil (21%), β-turn (13%) and β-sheet (9%). These data are in close agreement with a computational prediction of secondary structure from the primary amino acid sequence, which indicated 55.8% α-helix, 33.7% random coil and 10.5% β-sheet; the enzyme is, therefore, similar to the 4-chlorobenzoyl coenzyme A dehalogenase from *Pseudomonas* sp. CBS-3. The three-dimensional structure, including that of the presumed active site, predicted by computational analysis, is also closely similar to that of the *Pseudomonas* dehalogenase. Study of the stability and physicochemical properties revealed that at room temperature, the enzyme was stable for 24 h but was completely inactivated by heating to 60°C for 5 min; thereafter by cooling at 1°C min^{-1} to 45°C, 20.6% of the activity could be recovered. Mildly acidic (pH 5.2) or alkaline (pH 10.1) conditions caused complete inactivation, but activity was fully recovered on returning the enzyme to pH 7.4. Circular dichroism studies also indicated that secondary structure was little altered by heating to 60°C, or by changing the pH from 7.4 to 6.0 or 9.2. Complete, irreversible destruction of, and maximal decrease in the fluorescence yield of the protein at 330–350 nm were brought about by 4.5 M urea or 1.1 M guanidinium chloride. Evidence was obtained to support the hypothetical three-dimensional model, that residues W140 and W167 are buried in a non-polar environment, whereas W182 appears at or close to the surface of the protein. At least one of the enzymes of the dehalogenase system (the combined 4-chlorobenzoate:CoA ligase, the

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dehalogenase and 4-hydroxybenzoyl coenzyme A thioesterase) appears to be capable of association with the cell membrane.

Keywords *Arthrobacter* sp. TM-1 · Circular dichroism · 4-Chlorobenzoate · 4-Chlorobenzoyl CoA dehalogenase · Luminescence spectrometry · Protein denaturation

Abbreviations

CD	Circular dichroism
4-CB	4-Chlorobenzoate
4-CBCoA	4-Chloro-benzoyl CoA
GdmCl	Guanidinium chloride
4-HB	4-Hydroxybenzoate
4-HBCoA	4-Hydroxybenzoyl CoA

Introduction

Arthrobacter sp. strain TM-1 utilises 4-chlorobenzoate (4-CB) as a sole source of carbon. The dechlorination of 4-CB constitutes the initial reaction sequence in the dissimilative pathway, proceeding by the thioesterification of 4-CB to 4-chlorobenzoyl CoA (4-CBCoA), which is dehalogenated to yield 4-hydroxybenzoyl CoA (4-HBCoA), then de-esterified to release 4-hydroxybenzoate (4-HB). These reactions are catalysed by 4-CB:CoA ligase, 4-CBCoA dehalogenase, and 4-HBCoA thioesterase, respectively.

4-CBCoA Dehalogenase has been purified to homogeneity using sequential column chromatography on hydroxyapatite, DEAE-Sepharose, and Sephacryl S-200 (Zhou et al. 2004)[1], and is closely similar in primary structure to the 4-CBCoA dehalogenase of *Arthrobacter* sp. strain 4-CB1 (Crooks and Copley, 1994). Zhou et al. (2004) reported that the enzyme appeared to be a tetramer of identical 31 kD subunits with a molecular weight of 131 kD. Latterly, however, a re-determination by gel filtration of the molecular weight of the intact enzyme has yielded a value of 105.5 kD, which, taken together with a deduced molecular weight for the protomer of 29.9 kD (Gartemann et al. 1998), is not inconsistent with a trimeric structure.

Although 4-CBCoA dehalogenase from *Pseudomonas* sp. strain CBS-3 was initially also thought to be tetrameric (Chang et al. 1992), its structure determined by X-ray crystallography (Löffler et al. 1995; Benning et al. 1996) has revealed it to be a trimer. This enzyme has less, though significant, sequence similarity (46.5%) to that of strain TM-1 (Table 1). The best-studied of these dehalogenases, its reaction mechanism has also been deduced. D145 provides the side-chain carboxylate group that interacts with 4-CBCoA to form a Meisenheimer intermediate; H90 then serves as the general base in the subsequent hydrolysis step (Benning et al. 1996). Analysis of the dehalogenase-4-HBCoA complex has revealed that two H-bonds are contributed by the backbone amide protons of F64 and G114 to the C=O group of 4-HBCoA, and the G114A mutant form of the dehalogenase was shown to be strongly inhibited in both substrate binding and activation, indicating that H-bonding and/or interaction with the dipole of the 114–121 α-helix may be crucial. Another mutant form, W89Y, was inhibited in both catalysis and binding (Taylor et al.1995, 1997; Dong et al. 1999; Xiang et al. 1999).

The present report describes a physicochemical study of the dehalogenase of *Arthrobacter* TM-1, which was undertaken to ascertain structure/function relationships as a prelude to examining the reaction mechanism. Secondary structure, and the effect thereon of changes in temperature and pH, was investigated using circular dichroism (CD) and predictive computational approaches. The catalytic activity of the enzyme was investigated as a function of temperature, pH, and presence of the denaturants guanidinium chloride (GdmCl) and urea, and changes in enzyme conformation caused by these denaturants were investigated using fluorescence spectrometry.

Materials and methods

Enzyme purification and chemicals

4-CBCoA dehalogenase was purified to homogeneity from cells of *Arthrobacter* sp. strain TM-1 as described previously (Zhou et al. 2004). Urea and GdmCl, molecular biology grade, were purchased

[1] *Erratum:* The value reported for pI by Zhou et al. (2004) was incorrectly given as pH 6.42. The correct value is 5.70, compared with a predicted value of 5.54 given in UniProtKB/TrEMBL entry 085078 for the dehalogenase of *Arthrobacter* strain TM-1.

Table 1 Comparison of the peptide sequences[a] and secondary structures[b] of the 4-CBCoA dehalogenases from *Pseudomonas* CBS-3[c] and *Arthrobacter* strain TM-1[d]

```
                1
        c----   SEE  EEEETTEEEE  EE  GGGTT   B HHHHHHHH  HHHHHHHHTT  T  EEEEEES
        b----CCCCEE  EEEECCEEEE  EECCCCCCCC  CCHHHHHHHH  HHHHHHHHCC  CCCEEEEEEC
CBS-3:  ----MYEAIG  HRVEDGVAEI  TIKLPRHRNA  LSVKAMQEVT  DALNRAEEDD  SVGAVMITGA
        1       :       :::: :     :     :     :   : : ::  ::::   ::
TM-1:   MSSNSDHHIS  VEHTDGVATI  RFTRPSKHNA  ASAQLLLETL  EALYRLESDD  SVGAIVLTGE
        b CCCCCCCCEE  EEEECCEEEE  EECCHHHCCC  CCHHHHHHHH  HHHHHHHCCC  CCEEEEEECC

        TTBSB  B G  GGS SSSHHH  HHHHHHHHHH  HHHHHHHHHH  HH SSEEEEE  E SEEETHHH
        CCCCCCCCCC  CCCCCCCHHH  HHHHHHHHHH  HHHHHHHHHH  HHCCCCEEEE  ECCEEECHHH
CBS-3:  EDAFCAGFYL  REIPLDKGVA  GVRDHFRIAA  LWWHQMIHKI  IRVKRPVLAA  INGVAAGGGL
           : ::: :    : :          ::: :    :  :  ::     :   : :::  ::: : ::::
TM-1:   GAVFSAGFDL  EEVP-MGPAS  EIQSHFRLKA  LYYHAVIHML  ARIEKPTLAA  INGPAVGGGL
        CCCCCCCCCH  HHHC-CCCCC  CHHHHHHHHH  HHHHHHHHHH  HHCCCCEEEE  CCHHHHHHHH

        HHHHHSSEEE  EETT EEE    HHHHT    T  THHHHTHHHH  THHHHHHHHH  H   EEHHHH
        HHHHHCCEEE  EECCCEEECC  HHHHCCCCCC  CHHHHHHHHH  HHHHHHHHHH  HCCCCCHHHH
CBS-3:  GISLASDMAI  CADSAKFVCA  WHTIGIGNDT  ATSYSLARIV  GMRRAMELML  TNRTLYPEEA
        : ::: : :   : : : :  :  :  ::: ::    :  : :::  : :::::  :  :::::   ::
TM-1:   GMSLACDLAV  CTDRATFLPA  WMSIGIANDA  SSSFYLPRIV  GYRRAMEWLL  TNRTLGADEA
        HHHHHCCEEE  ECCCCEEECC  CCCCCCCCCC  HHHHHHHHHH  HHHHHHHHHH  HCCCCCHHHH

        HHHTS SEEE   HHHHTHHHH  HHHHHHHHTT  HHHHHHHHHH  HHHHTTTTHH  HHHHHHHHHH
        HHHCCCCCEE  CHHHHHHHHH  HHHHHHHCC  HHHHHHHHHH  HHHHCCCCHH  HHHHHHHHHH
CBS-3:  KDWGLVSRVY  PKDEFREVAW  KVARELAAAP  THLQVMAKER  FHAGWMQPVE  ECTEFEIQNV
          :: : ::       :          :: :::::  ::::    : :     :     :  ::: : :::
TM-1:   YEWGVVNRVF  SEADFQSRVG  EIARQLAAAP  THLQGLVKNR  IQEGSSETLE  SCTEHEVQNV
        HHCCCHHHCC  CHHHHHHHHH  HHHHHHHHCC  HHHHHHHHHH  HHHHHCCCHH  HHHHHHHHHH

                                                  269
        HHHHHTHHHH  HHHHHHHTTT  T    SS
        HHHHHCCCHH  HHHHHHHCCC  CCCCCCCCCC  CCC
CBS-3:  IASVTHPHFM  PCLTRFLDGH  RADRPQVELP  AGV
        :::: ::::     :   :                    276
TM-1:   IASVGHPHFA  ERLAMFRSKE  MRSSALAVDL  DAVCGGR
        HHHHCCHHHH  HHHHHHHHCC  CCCHHHHCCH  HHHCCCC
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[a] Bolded residues are important in catalysis in *Pseudomonas* CBS-3

[b] Secondary structure predictions were obtained from PSIPRED. C = coil; E = strand; H = helix

[c] Sequence and secondary structural data from 1NZY (Benning et al. 1996). B = isolated β-bridge; E = extended strand; G = 3_{10} helix; H = α-helix; S = bend; T = H-bond turn

[d] Sequence data for strain TM-1 from NCBI Accession No. AAF78820 (Gartemann et al. 1999)

from BDH Ltd (Poole, Dorset, UK). Unless otherwise stated, all other chemicals of both general and analytical reagent grade were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK), Fisher Ltd (Loughborough, Leics, UK), or Bio-Rad Laboratories (Watford, Herts, UK). Buffers used were 20 mM potassium phosphate, pH 5.2 and pH 7.4, and Clark & Lubs, pH 10.1 (50 mM potassium chloride/50 mM boric acid; pH adjusted with sodium hydroxide), all supplemented with 2 mM DTT.

Assay for 4-CBCoA dehalogenase activity

Enzyme activity was measured spectrophotometrically using a λ-2 spectrophotometer (Perkin-Elmer Corp., Beaconsfield, Bucks, UK), taking the difference in absorbance at 300 nm between 4-CBCoA (the substrate) and 4-HBCoA (the product). Enzyme assays were performed in 0.5 ml cuvettes at 30°C, in phosphate/DTT buffer, pH 7.4, and 7.5 µg of purified enzyme, in a total volume of 395 µl. Routinely, substrate (5 µl of 13 mM 4-CBCoA) was added to initiate the reaction, which was followed for 3–4 min (Zhou et al. 2004).

Stability of 4-CBCoA dehalogenase as a function of storage temperature

4-CBCoA dehalogenase in phosphate/DTT buffer, pH 7.4, or phosphate buffer, pH 7.4, lacking DTT (150 µl) was added to 50 µl of glycerol, to give a final concentration of 1.5 mg of protein ml^{-1}, and held at room temperature or 4°C. Control samples lacking glycerol were diluted to the same concentration with

more buffer with or without DTT, as appropriate. Samples containing 7.5 μg of enzyme were removed from these mixtures at regular intervals for up to 7 days thereafter, and activity was monitored as described above.

Determination of the extinction coefficient

Triplicate samples of purified dehalogenase in 20 mM-phosphate/2 mM-DTT buffer, pH 7.4, (20 μl) were dried under an infra-red lamp for 4 h, then placed in a desiccator over phosphoric oxide for 24 h. To allow for water regain, the samples were weighed repeatedly at 10 s intervals on a Sartorius microgram balance for 80 s, and the true dry weight was determined by extrapolation back to the time of removal from the desiccator. Correction for buffer dry weight was made by submitting buffer samples to the same procedure. Uv spectra of the enzyme in the same buffer were measured in a λ-2 dual beam recording spectrophotometer, against buffer alone, in 1-cm quartz cuvettes.

Circular dichroism (CD) studies

CD spectra of purified dehalogenase were recorded on a Model 62 DS spectropolarimeter (AVIV Associates, Inc., Lakewood, NJ, USA) interfaced with an IBM PC microcomputer for automatic data collection and analysis. Enzyme (1.5 mg ml^{-1}) was dissolved in phosphate/DTT buffer, pH 7.4, and centrifuged at 5000 rev min^{-1} for 5 min to remove particulate matter. Spectra were recorded between 260 and 190 nm in a 0.01 cm quartz cell. A computer program (CDNN v. 2.1; Boehm et al. 1992) was used to estimate the percentage of α-helix, β-sheet, β-turn and random coil in the molecule. This program assesses the contribution of each secondary structure by comparison of the experimental spectra with those of a set of proteins for which the secondary structural characteristics are fully known.

Denaturation of 4-CBCoA dehalogenase by GdmCl and urea

Stock solutions of 6 M GdmCl and 8 M urea were freshly prepared in phosphate/DTT buffer, pH 7.4, prior to each experiment (Schmid, 1989). Samples of dehalogenase were mixed, to make a final concentration of 1.5 mg ml^{-1}, with sufficient GdmCl or urea stock to yield a concentration range from 0 to 1.1 M GdmCl, or 0 to 4.7 M urea, at room temperature. Aliquots (5 μl) of these mixtures were withdrawn periodically for up to 60 min and diluted 80-fold prior to assaying the enzyme (7.5 μg) as described above.

Analysis by luminescence spectrometry

Mixtures (1 ml) were prepared containing 7.5 μg of purified enzyme and 0–1.3 M GdmCl, or 0–8.0 M urea, in phosphate/DTT buffer, pH 7.4. After 5 min, changes in the fluorescence spectrum of the enzyme at different denaturant concentrations were monitored in a Model LS-5B luminescence spectrometer (Perkin-Elmer) using an excitation wavelength of 280 nm (λ_{max} for tryptophan absorbance), and an emission wavelength range of 300–400 nm.

Renaturation of 4-CBCoA dehalogenase as a function of pH

Purified enzyme was mixed at a concentration of 1.5 mg ml^{-1} with phosphate/DTT buffer, pH 5.2 or 7.4, or Clark & Lubs/DTT buffer, pH 10.1, and held for 5 min; then 5 μl of the mixture (7.5 μg of enzyme) was withdrawn, returned to pH 7.4 and assayed for activity, as described above.

Renaturation of 4-CBCoA dehalogenase as a function of temperature

4-CBCoA dehalogenase (1.5 mg ml^{-1}) in phosphate/DTT buffer, pH 7.4, was denatured by heating at 60°C for 5 min, then cooled at 1°C per min to 55, 50, 45, 40, 30 or 20°C. Samples (5 μl; 7.5 μg of enzyme) were assayed at 45°C as described above, immediately after attaining the desired temperature.

Results

Stability of 4-CBCoA dehalogenase

Glycerol (25% v/v) exerted no effect on enzyme stability during storage either at 4°C or at room temperature. Both the 4-CBCoA dehalogenase from *Arthrobacter* sp. strain 4-CB1 (Crooks and Copley,

1994) and this one from strain TM-1, required stabilisation by DTT during and after purification, and remained fully active in its presence for 24 h at room temperature and for up to three days at 4°C. Without DTT, activity was quickly lost, suggesting the formation of a mixed population of oligomers containing some denatured enzyme. Both dehalogenases therefore contain free, labile –SH groups.

Extinction coefficient of the dehalogenase

Dehalogenase at a concentration of 1.235 mg ml^{-1} gave A = 0.52 at λ_{max} (280 nm), whence the extinction coefficient of the enzyme (29,899 D) was calculated to be 12.59 mM^{-1} cm^{-1}. This is 49.5% of the value (25.44 mM^{-1} cm^{-1}) predicted in UniProtKB/TrEMBL entry 085078 for *Arthrobacter* strain TM-1 dehalogenase, and presumably reflects quenching of the absorbance consequent on polypeptide folding and trimerisation of the subunits.

Secondary-structural analysis of 4-CBCoA dehalogenase

The secondary structure of the dehalogenase in phosphate/DTT buffer, pH 7.4, computed using program CDNN v. 2.1 (Boehm et al. 1992) from the far-uv CD spectrum (Fig. 1), consisted of 56% α-helix, 9% β-sheet, 13% β-turn and 21% random coil (Table 2). For purposes of comparison, after combining the β-turn and random coil contents (giving 34%) these data match closely the values of 55.8% α-helix, 10.5% β-sheet and 33.3% random coil obtained using PSIPRED v. 2.4 (McGuffin et al. 2000; Jones, 1999), which predicts secondary structure from the primary amino-acid sequence (Tables 1, 2).

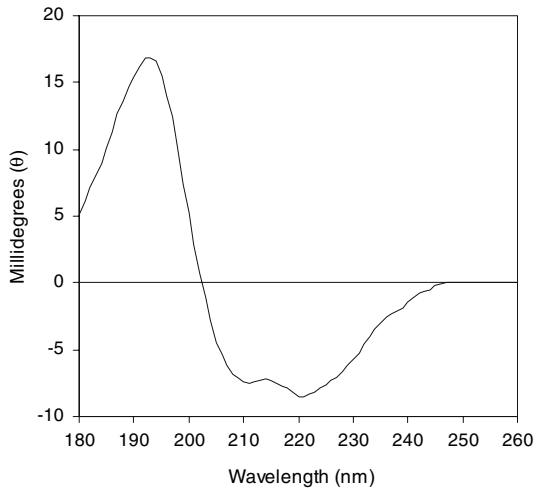

Fig. 1 CD spectrum of 4-CBCoA dehalogenase in the far uv region. The spectrum of purified enzyme (1.5 mg ml^{-1}) in phosphate/DTT buffer, pH 7.4, was measured in a 0.01 cm quartz cell

There is significant sequence similarity (46.5%) between the dehalogenases of *Arthrobacter* TM-1 and *Pseudomonas* CBS-3 (Table 1). Using X-ray crystallography, Benning et al. (1996) showed that the *Pseudomonas* enzyme is trimeric and consists of 52.8% α-helix, 14.9% β-sheet, and 32.3% random coil; similar values have been predicted using PSIPRED v. 2.4 (Table 2). Taking into account also the re-determined value for its molecular weight, the enzyme from TM-1 also appears to be trimeric rather than tetrameric (Zhou et al. 2004).

Using the SWISS-MODEL Protein Modelling Server (Guex & Peitsch, 1997; Peitsch, 1995; Schwede et al. 2003) a theoretical three-dimensional model (AAAaOI-Nk) of the dehalogenase from strain TM-1 was generated, matching the secondary structures of the two enzymes. The residues known to be involved in catalysis in the dehalogenase of strain CBS-3 (Table 1) were, except for W89, matched by identical residues in similar positions in strain TM-1 dehalogenase. Of the four cysteine residues per subunit, three (C125, C130 and C231; C273 was not mapped in the model) were well-spaced and therefore unlikely to form intra-chain disulphide bridges, consistent with the observed lability of the enzyme in the absence of DTT. None of these residues was close to the active site.

Denaturation of 4-CBCoA dehalogenase

A wide variety of physical parameters and solvent components have been used as protein denaturing agents (Tanford, 1968; 1970). The extent to which these agents perturb the native structure differs widely. Considering maximum denaturant concentrations and physical conditions, for globular proteins, the following series of denaturing capacity generally holds: GdmCl > urea > SDS > high temperature > pH extremes > high hydrostatic pressure (Creighton, 1989; Schmid, 1989).

Table 2 Comparison of the secondary structures of 4-CBCoA dehalogenases from *Arthrobacter* strain TM-1 and *Pseudomonas* strain CBS-3

Analytical method:	*Arthrobacter* strain TM-1			*Pseudomonas* strain CBS-3	
	Circular dichroism[a]		Prediction (PSIPRED)	X-ray diffraction[b]	Prediction (PSIPRED)
Component:					
Helix	56%		55.8%	52.8%	50.9%
Anti-parallel β-sheet	4%	9%[c]	10.5%	14.9%	13.4%
Parallel β-sheet	5%				
Random Coil	21%	34%[d]	33.3%	32.3%	35.3%
β-Turn	13%				

[a] Spectral data from circular dichroism were submitted to computational analysis using CDNN 2.1, and percentages are rounded to the nearest integer

[b] Data from Benning et al. (1996)

[c] The quantities of antiparallel and parallel β-sheet given by CDNN 2.1 should be totalled for comparison with the combined values given in the other columns

[d] The quantities of β-turn and random coil given by CDNN 2.1 should be totalled for comparison with the values for random coil given in the other columns

pH Effects

The pH optimum for dehalogenase activity was 7.4. When incubated for 5 min at 1.5 mg ml^{-1} in buffer at pH 5.2 or 10.1, enzyme activity was lost completely, but in both cases was fully recovered within 17 min following pH readjustment to 7.4 (Table 3). Enzyme inactivation under mildly acidic or basic conditions was therefore reversible. Examination by CD revealed no effect on the secondary structure by alteration of pH to 6.0 or 9.2, at which the enzyme displayed, respectively, 67% and 60% of its activity at pH 7.4 (Zhou et al. 2004).

Table 3 Renaturation of 4-CBCoA dehalogenase after return from pH 5.2 or 10.1 to pH 7.4[a]

Initial pH [b]	pH after adjustment	Incubation time (min) [c]	Activity (units mg^{-1})	Recovery (%)
7.4	N/A[d]	N/A	0.410	N/A
5.2	7.4	7	0.363	89
5.2	7.4	17	0.410	100
10.1	7.4	7	0.365	90
10.1	7.4	17	0.407	99

[a] Enzyme activity in 0.1 M phosphate/DTT buffer was maximal at pH 7.4, and zero at pH 5.2 or 10.1

[b] Enzyme was mixed at 1.5 mg ml^{-1} with phosphate/DTT buffer, pH 5.2 or 7.4, or Clark & Lubs/DTT buffer, pH 10.1, and held for 5 min; then 5 µl of the mixture (7.5 µg of enzyme) was withdrawn and assayed for activity at pH 7.4

[c] After returning to pH 7.4, samples were held at room temperature for 7 or 17 min before assay

[d] N/A = not applicable

Temperature effects

Activity was maximal at 45°C, and was totally lost by heating to 60°C for 5 min. After cooling at 1°C min^{-1} to 55, 50, 45, 40, 30 and 20°C, measurements at 45°C immediately thereafter showed that about one-fifth of the activity had been recovered during cooling within the temperature range down to 45°C (Table 4A). Thermal denaturation at low enzyme concentration was therefore partially reversible. At 45°C, the enzyme remained essentially stable for at least 2 h (Table 4B).

Heating the enzyme from 25 to 35, 45, 55 and 60°C produced no significant changes in the CD spectrum. In gross terms, therefore, the secondary structure appeared to be relatively unaffected by heating**.** It is unclear why the enzyme does not fully recover if the secondary structure is unaffected; possibly, under the conditions of the experiment, at 60°C sulphydryl groups have begun to undergo oxidation.

Effects of urea and GdmCl

Enzyme activity was unaffected by urea concentrations below 2.5 M (Fig. 2) but was completely lost

Table 4 Renaturation of 4-CBCoA dehalogenase following exposure to elevated temperature[a]

A.	Temperature (°C)	Activity (units mg^{-1})	Activity after heating to 60°C (units mg^{-1})	Recovery (%)
	60	0.000		
	55	0.194	0.081	20.1
	50	0.349	0.082	20.4
	45	0.402	0.083	20.6
	40	0.360	0.055	13.7
	30	0.301	0.051	12.7
	20	0.243	0.038	9.5

B.	Time elapsed at 45°C (min)	Activity (units mg^{-1})	Recovery (%)
	0	0.082	20.4
	5	0.082	20.4
	15	0.082	20.4
	120	0.080	19.9
	1020	0.007	1.7

[a] 4-CBCoA dehalogenase activity was maximal at 45°C, and was lost when the enzyme was heated at 60°C for 5 min. (A) Enzyme activity over the range 20–60°C, and recovery of activity after heating for 5 min at 60°C, followed by cooling at 1°C per min to the temperatures indicated, and immediate measurement of activity at 45°C. (B) Recovery of activity after heating for 5 min at 60°C, followed by cooling at 1°C per min to 45°C. Activity was measured at 45°C after incubation at the same temperature for the times indicated

after 5 min contact at 4.7 M. Irradiation of the native enzyme at 280 nm yielded the fluorescence emission spectrum shown in Fig. 3. Denaturation was accompanied by a steady decrease in the emission spectrum and a shift in the emission λ_{max} (Fig. 3) from 330.5 nm (native enzyme) to 344.5 nm in the presence of 7 M urea. In 2 M urea, at which the activity was still undiminished, fluorescence was already reduced by 30% of the final loss (Fig. 4), but was still only 70% complete at 4.7 M, when all activity was abolished. Both processes were 50% complete at approximately 3.0–3.5 M urea.

This enzyme contains only three tryptophan residues: W140, W167 and W182. For comparison, the single tryptophan residue of native ribonuclease absorbs light maximally at 280 nm and fluoresces maximally at about 319 nm, and this peak is 3.4 times more intense than the peak at 347 nm which is generated from the denatured protein by the now

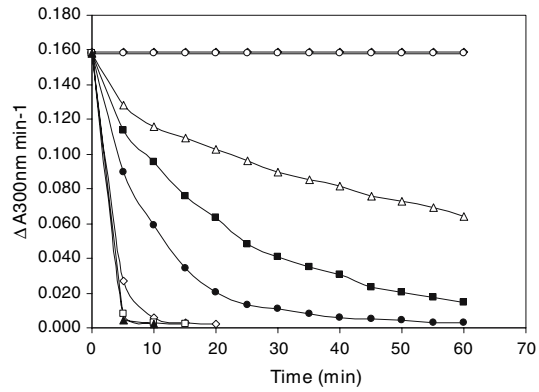

Fig. 2 Loss of 4-CBCoA dehalogenase activity as a function of urea concentration. 4-CBCoA dehalogenase was mixed, to make a final concentration of 1.5 mg ml^{-1}, with sufficient 8 M urea solution in phosphate/DTT buffer, pH 7.4, to yield a range of urea concentrations (0–4.7 M) at room temperature. Aliquots (5 µl) of the mixtures were withdrawn periodically for up to 60 min and diluted 80-fold prior to assaying the enzyme (7.5 µg) as described in the Experimental section. Reactions were initiated by the addition of 4-CBCoA. Changes in enzyme activity, expressed as $\Delta A_{300\ nm}\ min^{-1}$, are plotted against denaturation time. Urea concentrations (M): ♦, 0.0; ○, 2.0; Δ, 2.5; ■, 3.0; ●, 3.5; ◇, 4.0; □, 4.5; ▲, 4.7

exposed residue (Schmid, 1989). The theoretical model of the dehalogenase shows that W140 and W167 are buried in a non-polar environment, whereas W182 appears at or close to the subunit surface. The fluorescence yield for the native enzyme at 330.5 nm was 2.2-fold greater than the yield at 344.5 nm for the denatured enzyme. Therefore the intact enzyme plausibly contains two buried tryptophan residues and one at the enzyme surface, all of which are exposed to the aqueous (polar) exterior on complete denaturation, as the model predicts. The ratio of the fluorescence intensity from the buried tryptophan residues at 330.5 nm before denaturation (ignoring the contribution from the surface residue at that wavelength), to the value at 344.5 nm afterwards, might be expected to be 2.3:1. The fluorescence should therefore drop to 43.5% of the initial value; the observed drop was 45.5%.

As expected, the denaturant effect of GdmCl towards the dehalogenase was greater than that of urea. Inactivation of the enzyme (Fig. 5) was first apparent in 0.6 M GdmCl, and was complete after 10 min exposure to 1.1 M. Beginning at 0.7 M, the emission λ_{max} shifted steadily from 330.5 nm to a final value of 337.5 nm at 1.3 M GdmCl, with an

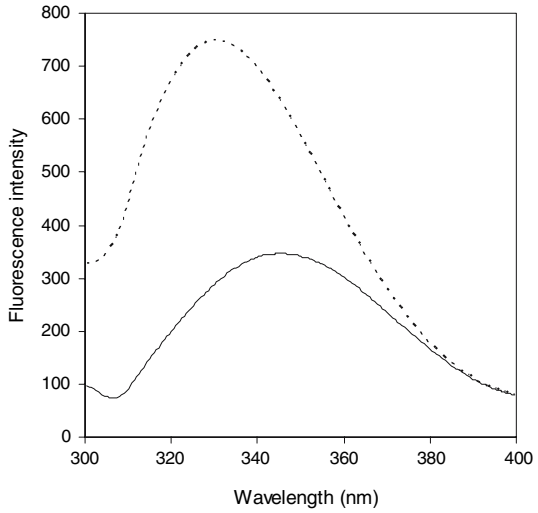

Fig. 3 Changes in the fluorescence emission of 4-CBCoA dehalogenase during denaturation in urea. Mixtures (1 ml) were prepared containing 7.5 μg of purified enzyme and 0 to 8.0 M urea, in phosphate/DTT buffer, pH 7.4. After 5 min, changes in the fluorescence spectrum of the enzyme at different urea concentrations were monitored by luminescence spectrometry (excitation wavelength 280 nm; emission wavelength range 300–400 nm). Dashed line, fluorescence spectrum of native enzyme; solid line, fluorescence spectrum of enzyme in 8 M urea

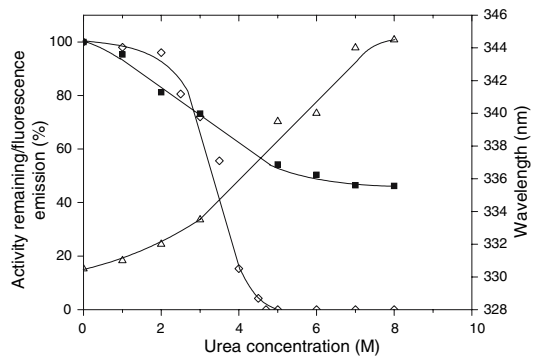

Fig. 4 Comparison of inactivation with changes in the fluorescence spectrum of 4-CBCoA dehalogenase in urea. Changes in enzyme activity and fluorescence emission over the first 5 min period, and in the fluorescence emission maximum, are plotted against urea concentration. ◇, Enzyme activity (% of the initial value); ■, fluorescence emission at λ_{max} (% of the initial value); Δ, λ_{max} of emission spectrum (nm)

overall drop in fluorescence of 2.3-fold (Fig. 6). Loss of activity was accompanied by a decrease in fluorescence emission, also first apparent at approximately 0.6 M and settling on a final value at 1.1 M GdmCl. Enzyme inactivation and the decrease in

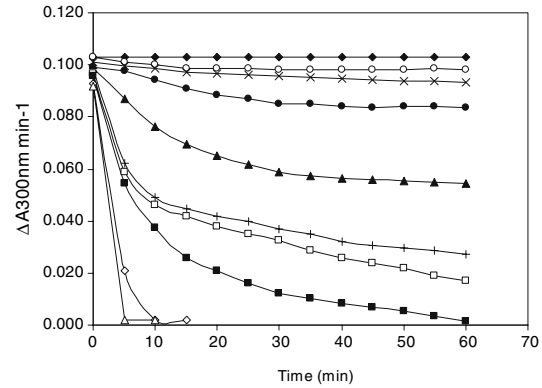

Fig. 5 Loss of 4-CBCoA dehalogenase activity as a function of GdmCl concentration. 4-CBCoA dehalogenase was mixed, to make a final concentration of 1.5 mg ml^{-1}, with sufficient 6 M GdmCl solution in phosphate/DTT buffer, pH 7.4, to yield a range of GdmCl concentrations (0–1.1 M) at room temperature. Aliquots (5 μl) of the mixtures were withdrawn periodically for up to 60 min and diluted 80-fold prior to assaying the enzyme (7.5 μg) as described in the Experimental section. Reactions were initiated by the addition of 4-CBCoA. Changes in enzyme activity, expressed as $\Delta A_{300\ nm}\ min^{-1}$, are plotted against denaturation time. GdmCl concentrations (M): ♦, 0.0; ○, 0.3; ×, 0.4; ●, 0.5; ▲, 0.6; +, 0.7; □, 0.8; ■, 0.9; ◇; 1.0; Δ, 1.1

fluorescence were approximately 50% complete at 0.8–0.9 M GdmCl (Fig. 7). The observed drop in fluorescence after denaturation with GdmCl was to 43.3% of the original value.

Enzyme incubated in the presence of 1 M GdmCl or 4 M urea at room temperature for 12, 24 and 48 h, was not reactivated within the duration of the experiment (about 10 min), after 80-fold dilution in phosphate/DTT buffer, pH 7.4 and immediate assay following the addition of substrate. The effects of urea and GdmCl on the CD spectrum of the enzyme have not been examined.

Possible membrane association of enzyme(s)

When first isolated, *Arthrobacter* TM-1 grew very slowly, but by selective subculture the doubling time (t_D) was gradually reduced from 50 h to 1.6 h (Marks et al. 1984; Marks, 1986). Increases in growth rate were matched by proportionate increases in the apparent specific activity of the dehalogenase system (the overall activity of the ligase, dehalogenase and thioesterase in combination), which implied faster growth made possible by the production of greater

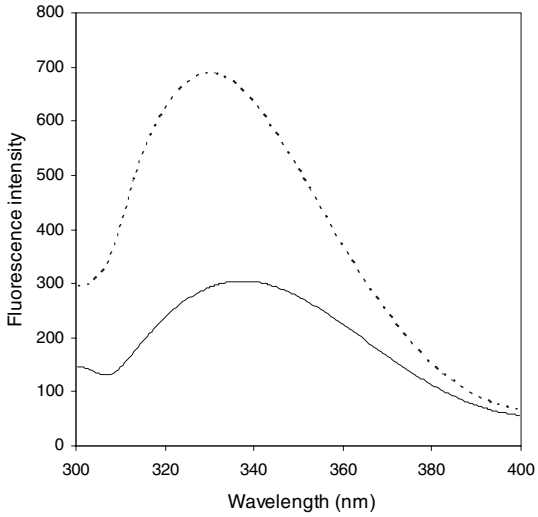

Fig. 6 Changes in fluorescence emission of 4-CBCoA dehalogenase during denaturation in GdmCl. Mixtures (1 ml) were prepared containing 7.5 μg of purified enzyme and 0–1.3 M GdmCl in phosphate/DTT buffer, pH 7.4. After 5 min, changes in the fluorescence spectrum of the enzyme at different GdmCl concentrations were monitored in a luminescence spectrometer (excitation wavelength of 280 nm; emission wavelength range of 300–400 nm). Dashed line, fluorescence spectrum of native enzyme; solid line, fluorescence spectrum of enzyme in 1.3 M GdmCl

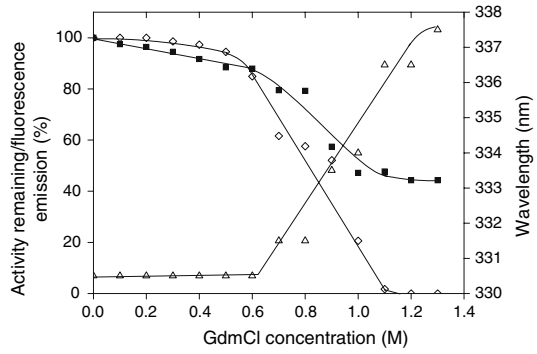

Fig. 7 Comparison of inactivation with changes in the fluorescence spectrum of 4-CBCoA dehalogenase in GdmCl. Changes in enzyme activity and fluorescence emission over the first 5 min period, and in the fluorescence emission maximum, are plotted against GdmCl concentration. ◇, Enzyme activity (% of the initial value); ■, fluorescence emission at λ_{max} (% of the initial value); Δ, λ_{max} of emission spectrum (nm)

amounts of these enzymes (Marks et al. 1984; Table 5). Freeze-press extracts of a variant strain with t_D = 20 h yielded, after ultracentrifugation, a cell membrane pellet beneath the soluble fraction, neither of which was enzymically active separately; but which regained activity on recombination (Marks et al. 1986). With further increases in growth rate, activity began to appear in the soluble fraction, and cells of t_D = 1.6 h gave extracts in which the activity resided almost entirely in the supernatant (Table 5). At least one of the enzymes of the dehalogenase system therefore *appears* to have been associated with the cell membrane, and conceivably, as the faster-growing variants emerged, there was insufficient surface area at the membrane to permit attachment of all the molecules; therefore the excess remained in the cytoplasm. Which enzyme(s) might be capable of membrane association remains to be determined.

Discussion

From the re-determined value for its molecular weight, the dehalogenase from TM-1 appears to be

Table 5 Relationship between specific activity of the dehalogenase system, specific growth rate of *Arthrobacter* TM-1, and contributions of the soluble and membrane fractions of cell-free extracts to the activity of the dehalogenase system

t_D (h)[a]	Specific growth rate (h^{-1})[a]	Specific activity of the dehalogenase system[a, b]	Enzyme activity (% of the value before centrifugation)[c]		
			Supernatant	Membrane	Recombined
20	0.034	5.4	0	0	100
12	0.058	8.4	20	0	100
1.6	0.433	51.0	100	0	100

[a] Data of Marks et al. (1984)

[b] nmol 4-HB formed h^{-1} (mg protein)$^{-1}$

[c] Data of Marks (1986). Cells grown on 4-chlorobenzoate were ruptured by freeze- pressing. After removal of intact cells by low speed centrifugation, extracts were ultracentrifuged at 100,000 × *g* for 2 h to yield a supernatant and a membrane pellet, which were tested for enzymic activity separately and in combination

trimeric rather than tetrameric (Zhou et al. 2004). The region in the dehalogenase from CBS-3 responsible for trimerisation (P206–Q262) is matched in TM-1 dehalogenase by the sequence P209–L265. These regions are 86% similar in their predicted secondary structure, displaying a high α-helix content, and may reasonably be expected to fulfil a similar function.

The theoretical model (AAAaOI-Nk) of the dehalogenase from strain TM-1 provided by the SWISS-MODEL Protein Modelling Server shows that it is possible to match its secondary structure with that of the dehalogenase of *Pseudomonas* CBS-3, and the residues known to be involved in catalysis in the latter are, except for W89, matched by identical residues in similar positions in strain TM-1 dehalogenase. W89 is one of three aromatic residues that form a hydrophobic ring around the bound ligand and participate in perpendicular stacking interactions with the 4-hydroxybenzoyl group (Benning et al. 1996). The W89Y mutant form of the *Pseudomonas* sp. CBS-3 dehalogenase is inhibited in both catalysis and binding (Taylor et al. 1995, 1997; Dong et al. 1999; Xiang et al. 1999), indicating a significant role in catalysis, but in *Arthrobacter* sp. strain TM-1, tyrosine positionally replaces this residue without affecting catalytic activity. When W89 is replaced by a tyrosine residue in the Swiss-Model of the CBS-3 enzyme, the plane of the tyrosine ring is seen to lie essentially in the same orientation as that of the tryptophan ring, but inspection of Y89 in the TM-1 enzyme model reveals that the plane of the ring is oriented approximately at 60° to that position. The significance of this difference to enzyme activity is clearly open to speculation, though possibly it explains why the enzyme from TM-1 can function despite the presence of a tyrosine residue at position 89.

The fall in fluorescence and shift in the emission λ_{max} in the presence of urea begin at low denaturant concentrations, but enzyme activity is unaffected up to 2.0 M, which suggests that some structural modification occurs initially without affecting catalytic activity. Other enzymes, e.g. papain, lose activity before conformational changes occur; the active site appears to be more flexible than the protein as a whole (Xiao et al. 1993; Wang et al. 1998). Above 2.0 M urea, all three processes follow in an apparently monophasic manner, which suggests that denaturation proceeds essentially in a single step.

GdmCl is in concentration terms a more effective denaturant than urea. In its presence, loss of activity and the fall in fluorescence begin gradually, but the emission λ_{max} remains unchanged up to 0.6 M GdmCl, therefore the unfolding mechanism apparently differs. Fig. 7 also shows possible inflexions in all three curves between 0.7 and 0.9 M GdmCl, indicating that the process may be more complicated. Unfolding in the presence of these denaturants often proceeds with the formation of an intermediate ''molten globule'', and the sequence with oligomeric proteins is potentially complex, as shown by Fan et al. (1998) in the case of the dimer creatine kinase. Inter-protomer disulphide and H-bonds, and hydrophobic and ionic interactions may also complicate the process (Yu et al. 1994).

Heat stability may permit the use of an enzyme at elevated temperature, a necessary attribute in an enzyme of potential industrial application such as the promotion of chlorine mineralisation; therefore knowledge of its stability is useful, because this is usually the factor that most limits the usefulness of enzymes. This dehalogenase is maximally active and shows a favourable level of stability at 45°C.

The enzyme may also be capable of association with the cell membrane, which warrants an exploration of its surface properties.

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