Biochemical and spectroscopic characterization of *Escherichia coli* aconitases (AcnA and AcnB)

Peter A. JORDAN^{†1}, Yue TANG^{*}, Alan J. BRADBURY^{*}, Andrew J. THOMSON[†] and John R. GUEST^{*2}

^{*}The Krebs Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, U.K., and

[†]Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

Escherichia coli contains two major aconitases (Acns), AcnA and AcnB. They are distantly related monomeric Fe–S proteins that contain different arrangements of four structural domains. On the basis of the differential expression of the *acnA* and *acnB* genes, AcnA has been designated as an aerobic-stationary-phase enzyme that is specifically induced by iron and oxidative stress, whereas AcnB functions as the major citric-acid-cycle enzyme during exponential growth. The biochemical and kinetic properties of the purified enzymes have now shown that AcnA is more stable than AcnB, has a higher affinity for citrate, and operates optimally over a wider pH range, consistent with its role as a maintenance or survival enzyme during nutritional or oxidative stress. In contrast, the better performance at high substrate concentrations and greater instability of AcnB indicate that AcnB is specifically adapted to function as the main catabolic enzyme and, by inactivation, to rapidly modulate energy metabolism in response to oxidative or pH stress, either directly or

indirectly by regulating post-transcriptional gene expression. EPR and magnetic-CD spectroscopy showed that the iron–sulphur clusters of the bacterial Acns (and their binding sites) strongly resemble those of the mammalian enzymes. The EPR and MCD spectra of the oxidized inactive form of AcnB confirmed the presence of a $[3\text{Fe-4S}]^{1+}$ ($S = 1/2$) cluster. Comparisons showed that the EPR spectrum of AcnB more closely resembled that of mammalian mitochondrial Acn (m-Acn), whereas the spectrum of AcnA more closely resembled that of the cytoplasmic enzyme (c-Acn). The MCD spectra revealed spectroscopic signatures similar to that of m-Acn. Reconstitution of the active $[4\text{Fe-4S}]^{2+}$ forms followed by one-electron reduction gave rise to EPR spectra that are almost identical with those reported for the mammalian enzymes.

Key words: citrate (isocitrate) hydrolyase, citric acid cycle, enzymology, EPR spectroscopy, iron–sulphur proteins.

INTRODUCTION

The aconitases (Acns), citrate (isocitrate) hydrolyases (EC 4.2.1.3), catalyse the reversible isomerization of citrate and isocitrate via the dehydration product *cis*-aconitate (see [1] for a comprehensive review). They function in the citric acid and glyoxylate cycles, and attention has recently been focused on their relation to the iron-responsive regulatory proteins that control vertebrate mRNA translation or stability [2–4]. Mammalian tissues contain a mitochondrial Acn (m-Acn) and a bifunctional cytoplasmic enzyme (c-Acn) which also functions as an iron regulatory protein (IRP1). They exhibit an overall amino acid sequence identity of 30%, and all of the residues considered to be essential active-site components, according to crystallographic and mutational studies, are conserved in both enzymes. The mitochondrial and cytoplasmic enzymes have very similar enzymic and other properties, but c-Acn is more sensitive to oxidation and has a recognizably different EPR spectrum [5]. The $[4\text{Fe-4S}]$ cluster, located at the site of enzyme activity, is unusual in being bound to the polypeptide chain by only three cysteine residues. In the absence of substrate, the fourth iron molecule, Fe_a , is associated with solvent hydroxide ion and, when substrate is added to the crystalline enzyme, isocitrate is bound to Fe_a via two oxygen atoms, one from the C α carboxy group and the other from the C α hydroxy group. A water molecule is also bound at Fe_a , making this a six-co-ordinate Fe site. In the absence of substrate under oxidative conditions, Fe_a is readily lost from the cluster, leaving a $[3\text{Fe-4S}]$ cluster bound

to the protein via the three cysteine residues. Upon reduction, the $[3\text{Fe-4S}]$ cluster has one less electron and it then regains an Fe(II) ion to regenerate the active site.

Crystallographic studies with pig heart mitochondrial Acn (m-Acn) have identified the presence of three structural domains (1–2–3) that are tightly packed around the iron–sulphur cluster and linked to a fourth domain (4) to form a deep active-site cleft [6]. This represents the basic structural archetype for all members of the Acn family, which includes other mono-functional Acns, the bifunctional cytoplasmic Acn/iron-regulatory proteins (c-Acn/IRP1), the mono-functional IRPs (IRP2), homoAcns and monomeric and heterodimeric isopropylmalate isomerases [7,8]. Two structural variations in which domain 4 is either located at the N-terminal end of the protein (as in some bacterial Acns) rather than the C-terminus, or exists as an independent subunit (as in bacterial isopropylmalate isomerases), have also been recognized [8].

Two genetically distinct Acns (AcnA and AcnB) have been identified in *Escherichia coli*, and the corresponding genes have been cloned and sequenced [9–11]. The presence of a weak residual Acn activity in an *acnA acnB* double mutant further suggests that *E. coli* possesses a third Acn, AcnC [12]. AcnA is more closely related to the bifunctional c-Acn/IRP1s (53% sequence identity) than to m-Acn (29% identity). In contrast, AcnB, which is approx. 17% identical with AcnA, m-Acn and the c-Acn/IRP1s, is the most remote member of the Acn family, even when the permuted domain organization (4–1–2–3 rather than 1–2–3–4) is taken into account. The active-site residues and

Abbreviations used: Acn, aconitase; m-, mitochondrial; c-, cytoplasmic; MCD, magnetic CD; IRP, iron-regulatory protein; IPTG, isopropyl β -D-thiogalactoside; DTT, dithiothreitol; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid.

¹ Present address: Department of Chemistry, University of Wyoming, Laramie, WY 82017-3838, USA.

² To whom correspondence should be addressed (e-mail j.r.guest@sheffield.ac.uk).

the cysteine residues that serve as ligands for the iron-sulphur cluster are conserved in both AcnA and AcnB. Clues about the physiological functions of AcnA and AcnB have come from studies with the corresponding *acn-lacZ* fusions and transcript analysis [13,14]. These showed that the *acnA* gene is expressed from a σ^{38} -dependent promoter and also from a σ^{70} -dependent promoter that is repressed by the transcription regulators FNR (fumarate and nitrate reduction regulator) and ArcA (aerobic respiration control regulator), and activated directly or indirectly by CRP (cyclic AMP receptor protein), Fur (ferric uptake regulator), iron, FruR (gluconeogenesis regulator) and SoxRS (superoxide stress regulator), whereas the *acnB* gene is expressed from a single σ^{70} -dependent promoter that is repressed by ArcA, FruR and Fis (factor for inversion stimulation), and activated by CRP. As a result it was concluded that AcnB is the major citric-acid-cycle enzyme, whereas AcnA is an aerobic-stationary-phase enzyme that is specifically induced by iron and oxidative stress. Recently, the AcnA and AcnB apoproteins have been shown to engage in site-specific mRNA binding and thus to regulate post-transcriptional gene expression, probably by via Fe-S-cluster-dependent switches [15].

The two most abundant *E. coli* Acns, AcnA and AcnB, have been purified from genetically amplified sources [11,16]. An EPR and magnetic-CD (MCD) spectroscopic analysis of AcnA revealed the presence of a $[3\text{Fe-4S}]^{1+}$ cluster and exhibited an EPR signal characteristic of a $[4\text{Fe-4S}]^{1+}$ cluster after reduction with sodium dithionite in the presence of Fe(II) ions, thus confirming that the expected cluster transformation occurs in the bacterial protein [16]. The present work describes a biochemical and spectroscopic characterization of AcnB, and comparisons with AcnA aimed at further defining the functional specificities of the major *E. coli* Acns, and identifying molecular adaptations that may be associated with their differential roles.

EXPERIMENTAL

Strains of *E. coli* K12 and plasmids

The isogenic derivatives of *E. coli* strain W3110 lacking AcnA (JRG2789, *acnA::kan^R*), AcnB (JRG3258, *acnB::tet^R*) and the double mutant (JRG3259, *acnA::kan^R acnB::tet^R*), have been described previously [8]. The Acns were purified from transformants of DH1 containing multicopy phagemids, pGS447 (*acnA*) in JRG2387 [9] and pGS801 (*acnB*) in JRG3171 [11], and from isopropyl β -D-thiogalactoside (IPTG)-induced cultures of BL21(DE3) strains containing pET21a expression plasmids, pGS1203 (*acnA*) in JRG4004 [15] and pGS783 (*acnB*) in JRG3099 [10].

Over production, purification, reconstitution and limited proteolysis of Acns

AcnA and AcnB were originally purified from the phagemid-containing strains, JRG2387 and JRG3171, by the methods described previously for AcnA [16] and AcnB [11]. The enzymes were later purified from shaken cultures (2×500 ml) of JRG4004 (*acnA*) and JRG3099 (*acnB*) grown to an attenuation/turbidity (*D*) at 595 nm of 0.5 at 37 °C, followed by enzyme induction with IPTG (1 mM) for 4 h at 25 °C (AcnA) or for 2 h at 37 °C (AcnB). The bacteria were harvested, resuspended in 20 ml of Tris/HCl buffer (40 mM, pH 8.0) and subjected to ultrasonic disruption. At this stage, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ (0.25 mM final concn.) and dithiothreitol (2.5 mM final concn.) were added to re-activate the Acns. Cell-free extract (150 mg of protein in 20 ml) was loaded on a DEAE-Sepharose column (16 mm $\times$ 35 mm) and washed with 5 vol. of Tris/HCl (40 mM, pH 8.0) before eluting Acn with

a linear gradient (300 ml, 0–0.2 M sodium citrate in the same buffer) using an FPLC system (Pharmacia). Coloured fractions containing AcnA or AcnB were pooled, and $(\text{NH}_4)_2\text{SO}_4$ (1.7 M final concn.) was added before applying to an Ether 650S column (16 mm $\times$ 40 mm). The column was washed with 5 vol. of 40 mM Tris/HCl, pH 8.0, containing 1.7 M $(\text{NH}_4)_2\text{SO}_4$, and Acn protein was eluted with a linear gradient [200 ml, 1.7–0 M $(\text{NH}_4)_2\text{SO}_4$ in the same buffer]. Typically about 30 mg of Acn protein (95% pure) was recovered. No significant differences were detected between the Acn proteins purified by different procedures.

Samples of purified AcnA and AcnB were reconstituted in an anaerobic workstation (Don Whitley Mk3) by adding $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ (140 μM), Na_2S (120 μM) and dithiothreitol (DTT) (1 mM) to Acn solutions containing 4 mg of protein/ml of 20 mM Tris/HCl, pH 8.0, and monitoring the reactions by UV-visible spectroscopy over the range 240–900 nm. The reconstituted enzymes were recovered by adding $(\text{NH}_4)_2\text{SO}_4$ (to 1.7 M) and repeating the final purification step using a small Ether 650S column (1 ml). The loaded columns were washed with 5 ml of 40 mM Tris/HCl, pH 8.0, containing 1.7 M $(\text{NH}_4)_2\text{SO}_4$ to remove free iron and sulphide, and then with 1 ml of salt-free buffer to elute Acn. In some cases, Acn was re-activated by incubating crude extracts with $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ (1 mM) and DTT (5 mM) in Tris/HCl (50 mM, pH 8.0) for 20 min at 20 °C.

Apo-AcnA and apo-AcnB were made by incubating Acn solutions with a 50-fold molar excess of EDTA, pH 8.0, and a 20-fold molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$ for 6 min at 4 °C as described by Kennedy and Beinert [17], and the protein was recovered using a small Ether 650S column as described above.

For limited tryptic proteolysis, 50 μg samples of AcnA or AcnB were treated with trypsin (0.1–1.0 μg trypsin in 20 mM Tris/citrate, pH 8.0) for 0, 5, 20 and 60 min at 37 °C. Digestion was halted by heating to 100 °C in SDS-loading buffer prior to polypeptide fractionation by SDS/PAGE [18], electroblotting to nitrocellulose using a Bio-Rad Transblot Electrophoretic Transfer Cell, and N-terminal amino-acid-sequence analysis using an Applied Biosystems protein sequencer.

Assays for Acn activity, protein, iron and acid-labile sulphide

The Acn activities of crude extracts and purified samples were assayed spectrophotometrically at 20 °C and pH 7.4 by monitoring the formation of *cis*-aconitate from isocitrate or citrate (20 mM) at 240 nm, using a molar absorption coefficient of 3600 M^{-1} [19]. One unit of activity corresponds to 1 μmol of *cis*-aconitate formed/min. The same units were used when monitoring the hydration of *cis*-aconitate (2 mM) at the same wavelength. The buffers used to vary the reaction mixture pH were (all at 20 mM): phosphate, pH 2 and 3; acetate, pH 4 and 5; Mes, pH 5–7; Tris, pH 7–9; and Caps [3-(cyclohexylamino)propane-1-sulphonic acid], pH 9–11.

Protein concentrations were estimated by the Bradford assay [20] using the Bio-Rad protein assay kit with BSA as standard. The iron contents of the purified enzymes and apoenzymes were assayed according to Woodland and Dalton [21] with modifications. Protein (10–15 nmol in up to 400 μl solution) was heated to 100 °C in trichloroacetic acid (10%, w/v) for 5 min to release the iron. Precipitated protein was sedimented and the supernatant fluid was transferred to a new Eppendorf tube and mixed with 30 μl of 1% bathophenanthroline-sulphonic acid, 0.4 ml of saturated sodium acetate and 90 μl of 20% fresh sodium ascorbate. After adjusting to 1 ml with water, the A_{535} was measured and compared with reference samples containing

$(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$. Acid-labile sulphide was assayed according to Beinert [22].

Spectroscopic methods

UV-visible absorbance spectra were obtained by scanning at 30 nm/min between 250 and 900 nm with a bandwidth of 1 nm using a Unicam UV4 spectrophotometer.

EPR spectra were recorded on an X-band Bruker ER-200D SRC spectrometer (perpendicular mode, 9.64 GHz) equipped with an Oxford Instruments (Oxford, U.K.) ESR-900 helium-flow cryostat and a TE-102 microwave cavity. Field intensity was monitored by a Hall probe and the microwave frequency was measured using a microwave counter (model 2440; Marconi Instruments, Stevenage, Herts., U.K.). Data were collected using a dedicated Bruker ESP-1600 computer. Spin densities of paramagnetic samples were estimated from integrations of EPR absorption spectra using 1 mM Cu(II)/10 mM EDTA as the standard solution [23].

Low-temperature MCD spectra were recorded on a JASCO J-500D spectropolarimeter interfaced to an IBM PC-AT computer. Glycerol (50 %, v/v; Aldrich spectroscopic grade) was used as a glassing agent. Samples were mounted in a split-coil superconducting magnet (SM4I; Oxford Instruments) capable of generating a magnetic field of 5 T with sample variation between 1.5 and 150 K. The CD spectra were measured with the same equipment, but without the magnetic field [24].

RESULTS

Relative abundance, reactivation and stability of AcnA and AcnB

The Acn activities of freshly prepared cell-free extracts of *E. coli* are generally lower than can be achieved after treatment with ferrous ions under reducing conditions, indicating that a significant proportion of the enzyme is in the inactive [3Fe-4S] or

apoenzyme forms. To investigate whether this is true for each of the three types of Acn detected in *E. coli*, extracts of late exponential-phase cultures of mutants lacking AcnA, AcnB and both AcnA and AcnB, were re-activated with ferrous ions under reducing conditions (Figure 1a). This showed that AcnB (the major component of the *acnA* mutant) and AcnA (the major component of the *acnB* mutant) can be re-activated. The residual activity of the *acnA acnB* double mutant (designated AcnC) was also re-activated to a small, but reproducible, extent, suggesting that AcnC also contains an iron-sulphur centre. After re-activation the specific activities of the mutants suggest that the overall activity of the parental strain is comprised of decreasing contributions from $\text{AcnB} > \text{AcnA} \gg \text{AcnC}$.

The stability and re-activation of AcnA and AcnB were studied with samples purified from genetically amplified sources which are essentially pure (> 95 %) after 5-fold enrichment. The preparation of both types of enzyme was facilitated by the use of two pET-derived *acn* expression plasmids, pGS1204 (*acnA*) and pGS783 (*acnB*), and a newly devised purification procedure based on ion-exchange chromatography rather than dye-affinity chromatography (see the Experimental section). Studies with typical preparations revealed three important differences between the two enzymes (Figure 1b). First, the starting activity of untreated AcnB was invariably much lower than that of AcnA, although both enzymes reached similar specific activities after re-activation. This indicates that, as prepared, AcnB contains a much higher proportion of inactive enzyme than AcnA. Secondly, the re-activation rate was 10-fold higher for AcnB than AcnA, the activity doubling times being 1.4 min (AcnB) and 14 min (AcnA) under the conditions used. Thirdly, after re-activation, AcnB decayed almost 7-fold faster than AcnA, the respective half-lives being 33 min (AcnB) and 210 min (AcnA). Thus it is clear that AcnB is considerably less stable than AcnA and it is also more readily re-activated. The lower stability of AcnB is probably due to the greater instability and enhanced degradation

Figure 1 Re-activation of the Acn activities of mutant extracts and purified enzymes

(a) The Acn activities of cell-free extracts of wild-type and mutant strains of *E. coli* were assayed at 30 min intervals before and after re-activation at with ferrous ions and DTT at pH 8.0: ◆, W3110 (wild-type parental strain); ■, JRG2789 *acnA::kan^R* (AcnB + C); ●, JRG3258 *acnB::tet^R* (AcnA + C); and ▲, JRG3259 *acnA::kan^R acnB::tet^R* (AcnC). (b) Purified Acn samples, AcnA (●) and AcnB (■), were assayed 15 min before re-activating with ferrous ions and DTT at pH 8.0, and then during the subsequent period of spontaneous inactivation. Acn specific activities units [(U)/mg of protein] were measured in Tris/citrate (20 mM, pH 8.0) at 20 °C.

Figure 2 pH profiles of AcnA and AcnB

Re-activated AcnA (●) and AcnB (■) were assayed in buffers (20 mM) of different pH. Overlap of the line occurs where two buffers of the same pH were used. The buffers were: phosphate, pH 2 and 3; acetate, pH 4 and 5; Mes, pH 5, 6 and 7; Tris, pH 7, 7.4, 8 and 9; and Caps, pH 9, 10 and 11.

of its iron–sulphur cluster at the pH (pH 8.0) used in the enzyme-stability tests (see below).

Optimum pH and kinetic characterization of AcnA and AcnB

The pH-versus-activity profiles of re-activated AcnA and AcnB showed that both enzymes have the same pH optimum, pH 7.4 (Figure 2). Furthermore, AcnA retained a high specific activity over a broad pH range, whereas the AcnB activity was highest over a narrower pH range, even though some variation in activity may be due to changes in ionic strength rather than pH. The broader activity range of AcnA might be a useful adaptation for an enzyme that is specifically expressed in the stationary phase, where the intracellular pH may vary over a wider range.

The specific activities of re-activated enzyme preparations varied over a 2-fold range regardless of the purification procedure. This is probably due to the presence of differing amounts of Acn protein that cannot be re-activated. Nevertheless, the apparent K_m and V_{max} values for citrate, *cis*-aconitate and isocitrate were determined for typical samples of purified AcnA and AcnB, using three methods to interpret the experimental data (Table 1). Significant differences between the kinetic properties of the two enzymes were observed, consistent with the view derived from their differential expression, that the two Acns are adapted for specific metabolic roles. In particular, the affinities of AcnA for citrate and isocitrate are 4–10-fold higher than those of AcnB but AcnA has a 3-fold lower affinity for *cis*-aconitate than AcnB, and the V_{max} values for AcnA with all substrates are 2–4-fold lower than those observed with AcnB (Table 1). Thus it would appear that AcnA is adapted to be the more effective at low concentrations of citrate or isocitrate, which is precisely what might be expected for a stationary-phase enzyme or one that can initiate or maintain citric-acid-cycle activity during metabolic stress. Likewise, the properties of AcnB are consistent with it being the major metabolic enzyme adapted to operate in exponential phase when the concentrations of citric-acid-cycle intermediates and carbon flux are likely to be high. It is also noteworthy that, at the higher concentrations of isocitrate, the affinities of both enzymes for this substrate are lowered some 50–100 fold, and although the maximum velocities increase, the overall catalytic activities (K_{cat}) are very significantly impaired (Table 1). The reason for this is not clear, but a similar situation has been observed with glutamate dehydrogenase and NH_3 [28].

Biochemical characterization and UV–visible spectroscopy of AcnA and AcnB

Typical samples of AcnA and AcnB were converted into apoenzymes by treatment with EDTA and ferricyanide and then reconstituted anaerobically with ferrous and sulphide ions (see the Experimental section). After cleaning up with Ether 650S, the apoenzymes contained negligible amounts of iron and sulphur, whereas the amounts of both elements in the holoenzymes

Table 1 Kinetic parameters of AcnA and AcnB

Kinetic parameters were determined for purified and re-activated samples of AcnA and AcnB using the spectrophotometric assay with various concentrations of citrate, *cis*-aconitate and isocitrate as substrates. Each assay was performed in duplicate and the values for K_m (mM) and V_{max} (units/mg of protein) were calculated using three types of plot (LB, Lineweaver and Burk [25]; EH, Eadie–Hofstee [26]; and ECB, Eisenthal and Cornish-Bowden [27]) with linear regression analysis (correlations in the range 0.996–0.900). The substrate concentrations were: citrate, 0.40–40 mM; *cis*-aconitate, 0.01–0.40 mM; and isocitrate, 0.01–40 mM. Two values were obtained for K_m and V_{max} with isocitrate as the substrate; the higher values correspond to those observed over the range 0.80–40 mM.

		AcnA			AcnB		
		K_m (mM)	V_{max} (units/mg)	K_{cat} (units/mg per mM)	K_m (mM)	V_{max} (units/mg)	K_{cat} (units/mg per mM)
Citrate	LB	1.16	6.13	5.28	11.0	23.8	2.16
	EH	1.15	6.14	5.34	11.2	24.2	2.16
	ECB	1.50	6.20	4.13	11.2	23.6	2.11
<i>cis</i> -Aconitate	LB	0.058	14.5	250	0.016	39.1	2444
	EH	0.053	13.9	262	0.018	40.9	2272
	ECB	0.060	14.5	242	0.020	42.0	2100
Isocitrate	LB	0.014; 1.77	3.57; 14.7	255; 8.30	0.051; 19.7	5.92; 57.8	116; 2.93
	EH	0.015; 1.24	3.79; 14.0	253; 11.30	0.052; 22.8	5.93; 63.8	114; 2.80
	ECB	0.015; 1.90	3.68; 15.0	245; 7.89	0.051; 24.5	5.92; 68.0	116; 2.78

Table 2 Iron and sulphur contents of AcnA and AcnB before and after reconstitution of the apoenzymes

Samples of AcnA and AcnB were converted into apoenzyme forms and then reconstituted anaerobically with ferrous and sulphide ions. The iron and sulphur contents and specific activities of the proteins were measured after treating with Ether 650S to remove unbound reactants.

Enzyme	Iron (atoms/mol of Acn)	Sulphur (atoms/mol of Acn)	Ratio Fe/S	Aconitase (units/mg of protein)
AcnA				
Apoenzyme	< 0.017	< 0.12	—	< 0.003
Reconstituted	3.64 ± 0.16	3.75 ± 0.30	0.97	15.7
AcnB				
Apoenzyme	< 0.017	< 0.12	—	< 0.003
Reconstituted	3.89 ± 0.09	3.34 ± 0.120	1.16	14.7

Figure 3 UV-visible spectra of the apo- and reconstituted forms of AcnA and AcnB

UV-visible absorption spectra over the range 250–900 nm are shown for samples of (a) apo-AcnA and (b) apo-AcnB, before (thin line) and after anaerobic reconstitution with ferrous and sulphide ions (thick line). The protein concentrations in 40 mM Tris/HCl, pH 8.0, were (mg/ml): apo-AcnA, 4.43; reconstituted AcnA, 5.01; apo-AcnB, 4.76; reconstituted AcnB, 5.13.

indicated that [4Fe–4S] clusters had been assembled in a major fraction of each enzyme (Table 2). The reconstituted enzymes regained catalytic activity, the specific activities being approximately the same for AcnA and AcnB (Table 2). The UV-visible absorption spectra of the same samples of the apo- and holo-enzymes are shown in Figure 3. The reconstituted enzymes are characterized by their higher absorbance in the 300–600 nm range and a prominent shoulder at 420 nm, which is typical for enzymes containing Fe–S clusters. Estimates of $\epsilon_{420}^{1\text{cm}}$ gave values of between 8×10^3 and $10 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in broad agreement with those reported for m-Acn [19,29].

Limited proteolysis was used to explore how the proposed differences in domain organization and linker length might affect the susceptibilities of the two bacterial Acns to digestion by trypsin. In AcnA (1–2–3–linker–4) it is predicted that the long linker between domains 3 and 4 is loosely coiled across the surfaces of domains 2 and 1, whereas in AcnB (4–linker–1–2–3) the linker provides a relatively short bridge between domains 4 and 1 [8,11]. Preliminary studies using different ratios of trypsin to protein with increasing times of incubation showed that AcnA is inherently more susceptible to proteolysis than AcnB. Treating AcnA with $0.01 \mu\text{g}$ of trypsin/ μg of protein released eight major

peptides within 5 min, whereas it took 60 min to release five detectable products from AcnB with the same amount of trypsin. Most of the AcnB products were large and had the same N-terminal sequence as the intact protein. Furthermore, the peptides released from the C-terminal end were too small, or present in insufficient quantity, to be characterized. The only trypsin-sensitive sites to be defined in AcnB were identified by the overlapping N-terminal sequences (KGKKNI and GKKN) of a 24 kDa product. They are located immediately distal to two lysine residues, K⁵⁶⁷ and K⁵⁶⁸ (one-letter amino acid code), in a sequence (TVEKKGKKNIFS) which is predicted to form a surface exposed loop that is uniquely inserted in domain 2 of AcnB [11]. The corresponding tryptic fragments should extend as far as the active-site arginine residues, R⁷⁹¹ and R⁷⁹⁶ in domain 3, near the C-terminus of AcnB. In the case of AcnA, the sizes (26–75 kDa) and/or N-terminal sequences of the digestion products identified several sites or potential sites of trypsin cleavage: K¹⁰⁸, R¹⁰⁹ and R¹⁶⁹ in two segments of random coil in domain 1; R³⁸³, K³⁹¹ and K⁴⁰⁶ in an AcnA-specific insert in domain 3; R⁶²⁷ in the linker; and R⁶⁸⁸, R⁶⁸⁹ or R⁷⁰⁷ in an unstructured region of domain 4 [10,11]. These observations show that AcnA is more vulnerable to tryptic proteolysis than

Figure 4 EPR spectra of the $[3\text{Fe-4S}]^{1+}$ states of AcnA and AcnB

(a) The EPR spectrum of AcnB (80 mM protein in 10 mM Mes, pH 6.0, and 5 mM MgCl_2) is compared with those of AcnA (replotted from [16]), c-Acn (replotted from [5]) and m-Acn (replotted from [5]). (b) EPR spectra of the $[3\text{Fe-4S}]^{1+}$ states of AcnB at pH 6.0 (10 mM Mes/5 mM MgCl_2) and pH 8.5 (50 mM Tris/25 mM KCl). The ordinate scales represent the first derivative of the EPR signal intensity with respect to the applied field (dI/dB)

AcnB and that the AcnA linker contains a major cleavage site. It would also appear that, even though the exact limits of the AcnB linker have not been defined, the greater resistance of AcnB is not due to the lack of trypsin-sensitive bonds in the linker but to its relative inaccessibility. This is because the polypeptide that is predicted to link potential secondary-structural elements in domains 4 and 1 contains several lysine and arginine residues [11].

EPR and MCD spectroscopy

The EPR spectra of the $[3\text{Fe-4S}]^{1+}$ clusters in AcnA and AcnB are compared in Figure 4 with those reported previously for the two mammalian enzymes, m-Acn and c-Acn [5]. The signals are centred at $g = 2.01$, but vary in shape for different proteins and also when glycerol is added to the aqueous buffer solvent. The signals arise from a single unpaired electron delocalized over the three Fe(III) ions in the clusters and they can be deconvoluted into three g values, if taken to be rhombic. The signals broadened as the temperature is raised above 30 K, as is the case for all known $[3\text{Fe-4S}]$ clusters. The overall forms of the EPR spectra (Figure 4a) suggest that closer similarities exist between the $[3\text{Fe-4S}]^{1+}$ clusters of AcnA and c-Acn and between those of AcnB and m-Acn. These relationships to some extent parallel similarities in the functional roles of the two pairs of enzymes, but this may be coincidental.

Comparing the EPR spectra of the $[3\text{Fe-4S}]^{1+}$ forms of AcnB at pH 6.0 and at pH 8.5 (Figure 4b) showed a dramatic loss of the signal at $g = 2.01$ and a marked increase in the signal at $g = 4.3$. These effects are reminiscent of cluster transformation into a linear form having a spin (S) of $5/2$. Such a transformation is well-documented for m-Acn, although in this case a pH value greater than 9.0 was required for cluster interconversion [31,32]. Thus it would appear that the $[3\text{Fe-4S}]$ cluster of AcnB has a narrower range of pH stability than that of m-Acn. Significant cluster degradation was also observed with samples of AcnB analysed at pH 8.0 in Tris buffer. Activity studies showed that the $[4\text{Fe-4S}]^{2+}$ form of AcnB also suffers substantial losses between pH 8.0 and 9.0 (Figure 2). Cluster instability may explain why AcnB has a rather narrow pH versus activity profile.

The low temperature MCD spectra of the $[3\text{Fe-4S}]^{1+}$ enzymes are complex (Figure 5). Comparing the MCD spectral forms of AcnA [16], AcnB and m-Acn [33] reveals minor variations in the relative intensities of different features, but the assignments of these spectra are not yet sufficiently advanced to allow the differences to be interpreted in terms of local structural variations around the respective clusters. Nevertheless, it is clear that the spectroscopic signatures of both bacterial enzymes resemble that of m-Acn [33]. No MCD spectra have been reported for c-Acn.

The $[4\text{Fe-4S}]^{2+}$ cluster is EPR-silent. However, treatment with a strong reductant such as sodium dithionite in the presence of Fe(II) ion an EPR signal characteristic of a $[4\text{Fe-4S}]^{1+}$ $S = 1/2$ cluster appears [29,30]. This signal is slow to develop, taking about 40 min to reach a maximum value. Complete reduction is often difficult to achieve with chemical reductants because the reduction potential is low. In the case of AcnA and AcnB the substrate-free forms have g -values of 2.05, 1.92, 1.85 and 2.05, 1.91, 1.86 respectively (Table 3; spectra not shown). These g -values are remarkably similar to those of the equivalent forms of c-Acn and m-Acn (see Table 3) and affirm the close similarity of the $[4\text{Fe-4S}]$ cluster binding sites in all four enzymes. In the presence of citrate the EPR g -values of c-Acn and m-Acn are shifted to values that denote an increase in rhombicity (Table 3). This is consistent with conclusions from other spectroscopies (including Mössbauer) that inequivalences arise between iron-

Figure 5 MCD spectra of the $[3\text{Fe-4S}]^{1+}$ states of AcnA and AcnB

The Figure shows the MCD spectrum of AcnB [160 mM in 50 mM Hepes, pH 7.8, and glycerol (50%, v/v) at 1.6 K, 1 mm path length and a magnetic field strength of 5 T] compared with that of AcnA (replotted from [16]).

Table 3 EPR parameters of $[4\text{Fe-4S}]^{1+}$ forms of aconitase

The g -values of several types of aconitase, rounded to three significant figures, were derived from: * [16]; † the present work; ‡ [5]; § [30].

Acn	Conditions	g -Values		
AcnA*	Mes/MgCl ₂ , pH 6.0	2.05	1.92	1.85
AcnB†	Mes/MgCl ₂ , pH 6.0	2.05	1.91	1.86
m-Acn‡	Hepes, pH 7.5	2.06	1.93	1.86
c-Acn‡	Hepes, pH 8.5	2.07	1.95	1.86
m-Acn + citrate§	Hepes, pH 7.5	2.04	1.85	1.78
c-Acn + citrate§	Hepes, pH 8.5	2.06	1.85	1.77

cluster sites upon substrate binding. No effects of citrate binding could be detected by EPR spectroscopy with AcnA or AcnB.

DISCUSSION

Consistent with their differential expression, relatively distant evolutionary relationships and presumed functional specializations, the two major Acns of *E. coli* exhibit significantly different biochemical and kinetic properties. The greater stability, wider functional pH range and greater affinity for citrate and isocitrate of AcnA are desirable features for an enzyme that is induced in stationary phase and in response to oxidative stress. As such it would better maintain a low level of metabolic activity for survival under conditions of nutritional and oxidative stress and likewise promote recovery when the stress abates. In contrast, AcnB appears to be better adapted for operating at higher

substrate concentrations as befits the most abundant exponential-phase enzyme. By virtue of its greater instability, AcnB would be better able to limit the damage that might ensue were a high metabolic rate to be maintained during oxidative stress. Also, in view of the recently discovered post-transcriptional regulatory activities of the bacterial Acns [15], AcnB would provide a particularly sensitive stress-responsive switch.

The EPR and MCD spectra of the oxidized inactive states of the bacterial Acns not only confirmed that they contain $[3\text{Fe-4S}]^{1+}$ clusters, as expected from their sequence similarities with mammalian Acns, they also point to a close overall similarity between the iron-sulphur clusters of the bacterial and mammalian enzymes. There are minor differences that cannot yet be interpreted in molecular detail. However, a strong correlation was established between enzyme activity and instability of the $[3\text{Fe-4S}]^{1+}$ iron-sulphur cluster of AcnB above pH 8.0, which undoubtedly explains why AcnB has a narrow pH versus activity profile.

Pairwise comparisons of the EPR spectra revealed that the spectral similarities are greater for AcnA and c-Acn and for AcnB and m-Acn than for the alternate pairings of bacterial and mammalian enzymes. While these relationships may be fortuitous, it is noteworthy that the roles deduced for the bacterial Acns identify AcnB as the functional homologue of m-Acn, whereas AcnA, which is 50% identical with c-Acn/IRP1, resembles the cytoplasmic enzyme in having an iron- and oxidative-stress-dependent function. Activation of the bacterial enzymes by transformation to the $[4\text{Fe-4S}]^{2+}$ forms was also shown by measuring the EPR spectra of the one-electron-reduced forms, $[4\text{Fe-4S}]^{1+}$. However, no EPR spectroscopic changes comparable with those associated with citrate binding to reduced active-site

clusters in the mammalian enzymes, were detected with the corresponding form of the bacterial enzymes.

The reason why *acnA*, the gene encoding the least abundant of the major *E. coli* Acns, was the first to be cloned, can now be explained in terms of the greater stability of AcnA relative to AcnB. The cloning procedure involved purifying Acn from a wild-type strain of *E. coli* and using the corresponding antiserum to screen λ -*E. coli* gene libraries for immunopositive *lacn* phages [9]. Inevitably the most stable, rather than the most abundant, enzyme was purified and the corresponding gene (*acnA*) was recovered.

Studies with the *acn* mutants indicate that at least one other Acn (designated AcnC) accounts for up to 5% of the parental Acn activity, and its re-activation by ferrous ions under reducing conditions suggests that it is an iron-sulphur enzyme. Whether it is an Acn *per se* that might be induced to higher activities under specific physiological conditions, or some other enzyme that has a substrate specificity broad enough to include citrate and isocitrate, is currently being investigated by enzyme purification. Potential candidates representing each of the above categories are: the product of the *ybhJ* gene [34], which is an unidentified paralogue of AcnA, and a potential 2-methylcitrate hydrolyase (2-methyl-Acn), which might be associated with the methylcitrate pathway of propionate catabolism in *E. coli* [35]. The former possibility is particularly interesting, because it might explain an earlier observation that Acn activity is amplified up to 18-fold after inducing lysogens containing members of a specific group of recombinant λ -phages [36]. These phages might be λ ybhJ or alternatively, because the *ybhJ* gene is almost immediately adjacent to the site of prophage integration (*att* λ) in the *E. coli* chromosome, the recombinant phages may have inserts that specifically promote phage-mediated transcription of the flanking chromosomal *ybhJ* gene.

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