**Sulfolobus aconitase, a regulator of iron metabolism?**

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**Abstract**

The aconitase of *Sulfolobus solfataricus*, a hyperthermophilic crenarchaeon, was cloned and heterologously expressed in *Escherichia coli*. Enzymic analyses and EPR measurements indicated clearly that the iron-sulphur cluster of the thermophilic aconitase was already inserted in the mesophilic host. The enzyme was purified to a specific activity of approx. 44 units/mg and to 90\% homogeneity. The enzymic parameters of the recombinant aconitase turned out to be in the same range as the respective values for the previously characterized native enzyme from the closely related *S. acidocaldarius*. Based on its primary sequence, the recombinant aconitase is closely related to bacterial A-like and to eukaryotic iron regulatory protein-like proteins. Specific aconitase activities in cytosolic extracts of *S. acidocaldarius* were found to be decreased markedly in iron-starved compared with iron-repleted cells. However, no differences in aconitase levels between iron-starved and iron-supplemented cells could be detected by immunostaining.

**Introduction**

With few exceptions, virtually all known organisms from Archaea to humans are dependent on iron for survival. In spite of its essential nature, the redox capacities of iron underlie its high potential toxicity. Consequently, organisms have evolved complex mechanisms for the tight regulation of iron uptake, storage and utilization.

In Bacteria, iron homoeostasis is triggered mainly at the transcriptional level through the actions of Fur (ferric uptake repressor)- and DtxR (*Diphtheria* toxin repressor)-like regulators (reviewed in [1]), whereas in vertebrates intracellular iron concentrations are controlled largely posttranscriptionally by iron regulatory proteins (IRPs). IRPs exert their effects by high-affinity binding to so-called iron responsive elements, stem–loop structures that are located in the untranslated regions of mRNAs coding for the regulated proteins (for reviews, see [2,3]). Interestingly, IRP1 is identical with the cluster-free apoform of vertebrate cytoplasmatic aconitase (EC 4.2.1.3), which belongs to a widely distributed and phylogenetically closely related class of enzymes that employs an interconvertible, redox-inactive [4Fe–4S] iron–sulphur cluster for catalytic activity (reviewed in [4,5]). The switch between posttranscriptional regulator and enzyme is mediated through the assembly/disassembly of the iron–sulphur cluster [2–4]. Although best characterized in Mammalia, recent work has demonstrated IRP-like functions of c-type (cytoplasmic type) aconitases in a variety of organisms, ranging from lower invertebrates to Bacteria [1,2,6]. In fact, nothing is known about iron regulation in Archaea.

Recently, we published the first purification and characterization of an archaeal aconitase [7].

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Key words: Archaea, heterologous expression, iron–sulphur cluster, thermophilic aconitase.

Abbreviations used: IRP, iron regulatory protein; c-type, cytoplasmic type.

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By sequence alignments of internal peptides, the enzyme of the hyperthermophilic *Sulfolobus acidocaldarius* could be clearly identified as a member of the c-type aconitase family [7]. After establishing its function in both the citric acid cycle and the glyoxylate cycle [7,8], we began to investigate its possible role as an iron regulator. Since the endogenous enzyme of *S. acidocaldarius* could not be purified to homogeneity, we decided to clone and heterologously express the putative aconitase of the closely related *S. solfataricus* for further investigations, because the complete genomic sequence of this organism has become available recently [9]. Here we describe for the first time the heterologous expression of an archaeal aconitase and a brief characterization of the recombinant protein. Furthermore, the possible role of *Sulfolobus* aconitase as an iron-sensing regulator is discussed.

**Results and discussion**

**Cloning and heterologous expression**

The gene encoding *S. solfataricus* P1 aconitase (2568 bp) was amplified by PCR, yielding a single specific product of approx. 2.6 kb, which was cloned in the expression vector pET-11a. With the exception of four point mutations, the aconitase gene of *S. solfataricus* P1 was identical with the respective sequence (Sso1095) deposited in the *S. solfataricus* P2 database (http://www-archbac.u-psud.fr/projects/sulfolobus/). To overcome the significant differences in the codon usages of *S. solfataricus* and the mesophilic host, the expression construct was transformed in *Escherichia coli* BL21 CodonPlus DE RIL (Stratagene). In spite of the use of this strain, the levels of expression of the recombinant protein under all conditions tested remained low, accounting for approx. 1–2.5 % of the soluble protein fraction. However, in anaerobically prepared cytosolic extracts of isopropyl β-D-thiogalactoside-induced cells, high specific aconitase activities were detectable after heat inactivation of the endogenous enzymes of *E. coli*. These data demonstrate unequivocally that part of the recombinant thermophilic aconitase was folded correctly and that the activity-determining iron–sulphur cluster was already inserted in the mesophilic host, even at a temperature that was 45 °C lower than the optimal growth temperature of *S. solfataricus*. This conclusion was further supported by EPR spectroscopy. It is not clear if the insertion is mediated by the iron–sulphur cluster machinery of *E. coli* (for review, see [10]) or occurs spontaneously. However, recent studies with oxidatively inactivated native aconitase from *S. acidocaldarius* have shown that the thermophilic enzyme could be reactivated at room temperature under reducing conditions in the presence of ferrous iron and sulphide [7].

**Purification and characterization**

The recombinant aconitase was purified to a specific activity of approx. 44 units/mg and to 90 % homogeneity by a semi-anaerobic three-step procedure (Figure 1). The N-terminal sequence of the recombinant protein (MPNKFSYKG) matches exactly the corresponding sequence of the putative aconitase of *S. solfataricus* P2 (see http://www-archbac.u-psud.fr/projects/sulfolobus/); therefore contamination with the enzymes of *E. coli* could be ruled out. The molecular mass of approx. 96 kDa is in excellent agreement with the values determined for the enzyme of *S. acidocaldarius* and other members of the c-type aconitase family by SDS/PAGE [5,7]. The *Sulfolobus* enzymes were comparable with respect to their $K_m$ values for citrate (360 ± 30 and 704 ± 90 μM) and cis-aconitate (53 ± 6 and 173 ± 35 μM), and their temperature (75.0 and 77.5 °C) and pH (7.4 and 7.0) optima. Like the native aconitase, the recombinant enzyme is remarkably resistant to thermal inactivation. Under strictly anaerobic conditions, its catalytic activity re-

![Figure 1](image-url)
mained unaffected even after a 1 h incubation at 80 °C.

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Iron availability was found to be critical for growth of *S. acidocaldarius*. Cells grown in media supplemented with 100 μM iron reached significant higher absorbance values and grew faster than iron-starved cultures in media without iron supplementation. The growth-limiting effect could be enhanced further by the addition of 25 μM bathophenanthroline disulphonic acid, an impermeant ferrous iron chelator, to iron-depleted media. However, supplementation of iron-depleted media with ≥ 40 μM chelator abolished growth, indicating a critical lower iron limit for *S. acidocaldarius*. Interestingly, the specific aconitase activities in the respective anaerobically prepared cytosolic extracts correlated well with iron availability in the medium. Activity was found to be decreased to approx. 25 % in cells grown in the presence of chelator as compared with the corresponding values in extracts of iron-repleted cells.

In contrast, the specific activity of endogenous isocitrate dehydrogenase, an iron-independent enzyme, was only marginally different in iron-repleted and iron-starved cells (Figure 2A). Western blot analyses of the various extracts revealed no differences in aconitase protein levels (Figure 2B). This result clearly indicates that a significant portion of the crenarchaeal iron–sulphur enzyme became inactivated under conditions of iron limitation. It is not known whether the iron–sulphur clusters of the inactive aconitase species decayed completely, the prerequisite for known IRP-like functions, or were converted into the catalytically inactive [3Fe–4S] state of the enzyme [2–4]. In this respect, it is of interest that the superoxide dismutase of *S. acidocaldarius*, one of the main cellular defences against the lethal effects of molecular oxygen, is an iron-dependent enzyme [11]. The question of whether the crenarchaeal aconitase, which shares more than 48 % sequence identity with human IRP1, may function as an iron- or oxygen-sensing regulator in *Sulfolobus* will be answered by future work.

**Figure 2**

**Effect of iron status on aconitase activity and levels** (A) Specific activities of aconitase and isocitrate dehydrogenase in anaerobically prepared cytosolic extracts of *S. acidocaldarius* cells grown in iron-supplemented medium (+Fe), without iron supplementation (−Fe) or in iron-depleted medium in the presence of 25 μM bathophenanthroline disulphonic acid (Bpt). Values are means ± S.D. of two individual cultures. (B) Determination of aconitase (Aco Sac) levels in cytosolic extracts of iron-supplemented and iron-starved *S. acidocaldarius* cells by immunostaining with a polyclonal antiserum against recombinant aconitase from *S. solfataricus*. Approx. 75 μg of protein was added to each lane; circles in lane 1 indicate the positions of the marker proteins.

**References**


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