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Role of the Heme Regulatory Motif in the Heme-Mediated Inhibition of Mitochondrial Import of 5-Aminolevulinate Synthase

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5-Aminolevulinate synthase (ALAS) is a mitochondrial enzyme that catalyzes the first step of the heme biosynthetic pathway. The mitochondrial import, as well as the synthesis, of the nonspecific isoform of ALAS (ALAS1) is regulated by heme through a feedback mechanism. A short amino acid sequence, the heme regulatory motif (HRM), is known to be involved in the regulatory function of heme. To determine the role of the HRM in the heme-regulated transport of the nonspecific and erythroid forms of ALAS *in vivo*, we constructed a series of mutants of rat ALAS1, in which the cysteine residues in the three putative HRMs in the N-terminal region of the enzyme were converted to serine ones by site-directed mutagenesis. The wild-type and mutant enzymes were expressed in quail QT6 fibroblasts through transient transfection, and the mitochondrial import of these enzymes was examined in the presence of hemin. Hemin inhibited the mitochondrial import of wild-type ALAS1, but this inhibition was reversed on the mutation of all three HRMs in the enzyme, indicating that the HRMs are essential for the heme-mediated inhibition of ALAS1 transport in the cell. By contrast, exogenous hemin did not affect the mitochondrial import of the erythroid-specific ALAS isoform (ALAS2) under the same experimental conditions. These results may reflect the difference in the physiological functions of the two ALAS isoforms.

Key words: aminolevulinate synthase, heme, mitochondrial import.

Abbreviations: ALAS, 5-aminolevulinate synthase; HRM, heme regulatory motif; TTBS, Tris-buffered saline containing 0.1% Tween-20; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

5-Aminolevulinate synthase (ALAS) catalyzes the condensation of glycine and succinyl-CoA to form 5-aminolevulinate in mitochondria, the first step of the heme biosynthetic pathway, and plays a central role in this pathway (*1*, *2*).

In mammals, two genes encoding ALAS are known. One, which is specifically expressed in erythroid cells (ALAS2), is responsible for synthesizing the large amount of heme required for hemoglobin synthesis. The other, which is expressed in all tissues (ALAS1), satisfies the basal requirement of heme in the cell (*3*).

Heme, the final product of the pathway, regulates the cellular expression and localization of these two isozymes (*2*). Although it is known that the transcription (*4*), translation (*5*), and mitochondrial import (*6*, *7*) of ALAS1 are regulated through a feedback process by heme, the precise mechanism by which this regulation takes place has not been clarified as yet. By contrast, the translational regulation of ALAS2 has been studied extensively. The iron-responsive element in the 5′-untranslated region of mRNA of ALAS2 has been shown to play a role in inhibiting mRNA translation (*8*). In erythroid cells, heme is necessary for mRNA translation and is important in erythroid differentiation (*9*). HAP-1, a transcription factor in yeast, possesses conserved, short amino acid sequences containing the dipeptide Cys-Pro (*10*). These so-called "heme regulatory motifs" (HRMs), which are found in such proteins as ALAS2 and erythroid-specific eIF2α kinase, are essential in heme-mediated regulation (*11–13*). Recently, Ogawa *et al*. showed that heme binds specifically to the dipeptide Cys-Pro motifs in Bach1 and thereby regulates the DNA-binding activity of this protein (*14*).

Mammalian ALASs contain three conserved motifs: two in the presequence and one in the mature form of the enzyme. Lathrop and Timko have shown that the presence of at least one such motif in the presequence enables heme to inhibit the mitochondrial import of mouse ALAS2 in *in vitro* experiments (*15*). However, it is not clear whether HRM regulates the mitochondrial import of ALAS *in vivo*, or whether the actions of HRMs differ between ALAS2 and ALAS1.

In this study, we examined the effect of mutations in the three HRMs present in the precursor of rat ALAS1. By changing the essential cysteine in each HRM to serine, we constructed all HRM mutant combinations. The mutant and wild-type enzymes were expressed in quail fibroblasts, and the relationship between the HRMs and heme inhibition of the mitochondrial import of ALAS1 was examined. We found that the HRMs were required

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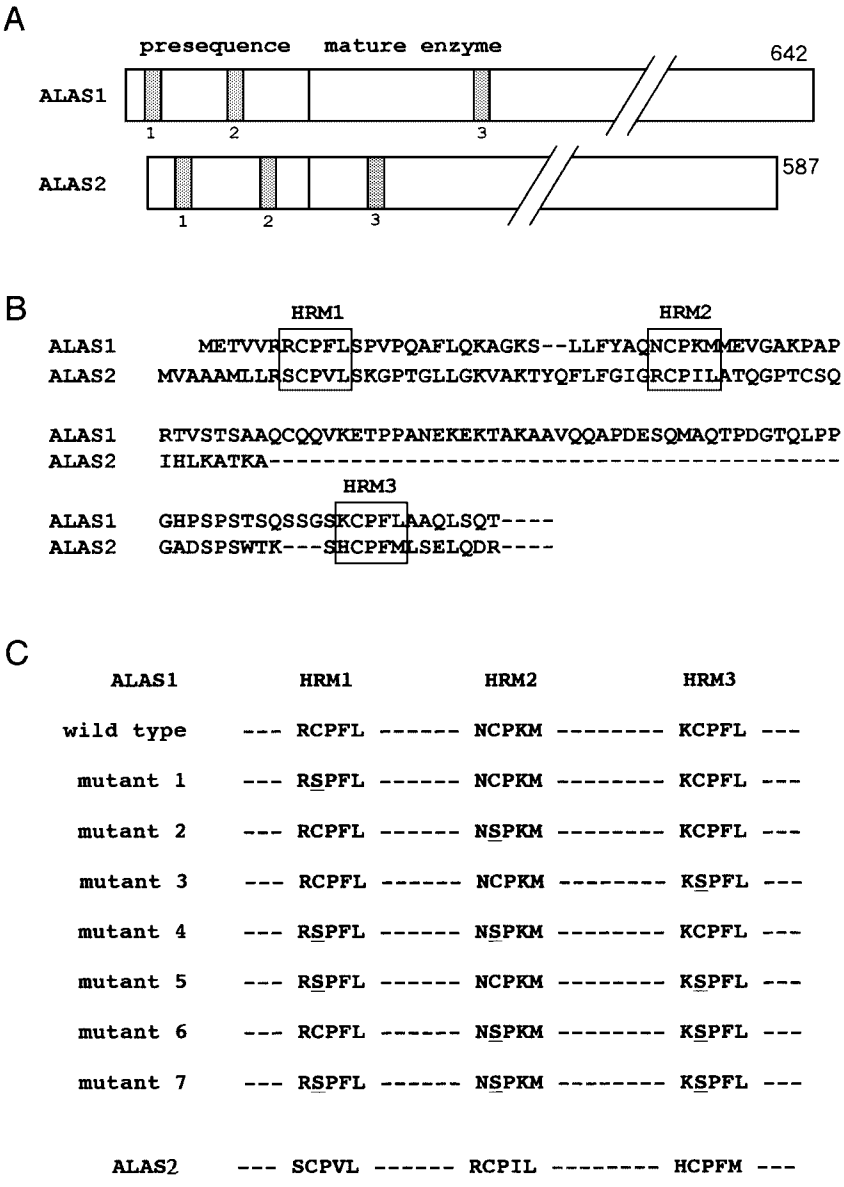

Fig. 1. **Site-directed mutagenesis of rat ALAS1.** A: Location of HRMs in ALAS1 and ALAS2 is depicted as shaded boxes. 1, HRM 1; 2, HRM 2; 3, HRM 3. B: Amino acid sequences of the ALAS1 and ALAS2 N-terminal regions. HRMs were boxed. C: A series of seven mutants (mutants 1–7) was prepared, in which the conserved cysteine residues in the three HRMs (HRM1, HRM2 and HRM3) were converted to serine ones by site-directed mutagenesis. The underlined residues indicate the positions of the mutations. Also shown are the HRMs in rat ALAS2.

for the heme-mediated inhibition of mitochondrial import of ALAS1. By contrast, under the same conditions heme showed no effect on the mitochondrial import of rat ALAS2. These results suggest that ALAS2 and ALAS1 are controlled by different heme regulation mechanisms *in vivo*, and that the HRMs in the two isoforms of ALAS do not perform the same function.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Site-Directed Mutagenesis—Rat ALAS1 cDNA cloned into pBluescript (*16*) was used to construct mutants. The site-directed mutants were constructed according to the Transformer Site-Directed Mutagenesis Kit manual (Clontech). The synthetic oligonucleotides used for site-directed mutagenesis were as follows: 5′-CGTTCGCAGATCCCCATTCTTAT-3′ (HRM1), 5′-TGCTCAAAACTCCCCCAAGATGA-3′ (HRM2), and 5′-TGGGAGCAAGTCCCCTTTCCTGG-3′ (HRM3), which correspond to nucleotides 37–8, 111–33, and 342–64 of rat ALAS1 cDNA, respectively, in which the underlined nucleotides were altered. The rat ALAS2 cDNA has been described previously (*17*). The wild-type and mutated rat ALAS1 cDNAs, and rat ALAS2 cDNA were digested with *Eco*RI and then inserted into the unique *Eco*RI site of eukaryotic expression vector pCAGGS (*18*).

Transfection and Expression of ALAS—Quail fibroblast cell line QT6 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and then seeded into 12-well dishes 24 h before transfection. QT6 cells were transfected with a vector encoding the cDNA of ALAS2, and the wild-type and mutant ALAS1 were subcloned into pCAGGS by the calcium phosphate precipitation method. The medium was changed after 12 h and, at the same time, the indicated amount of hemin was added to each well. The QT6 cells were harvested 6 h after the addition of hemin.

Isolation of Mitochondria—QT6 cells were transfected with the cDNAs of ALAS1 as described above. The medium was changed at 12 h, and after 6 h the QT6 cells

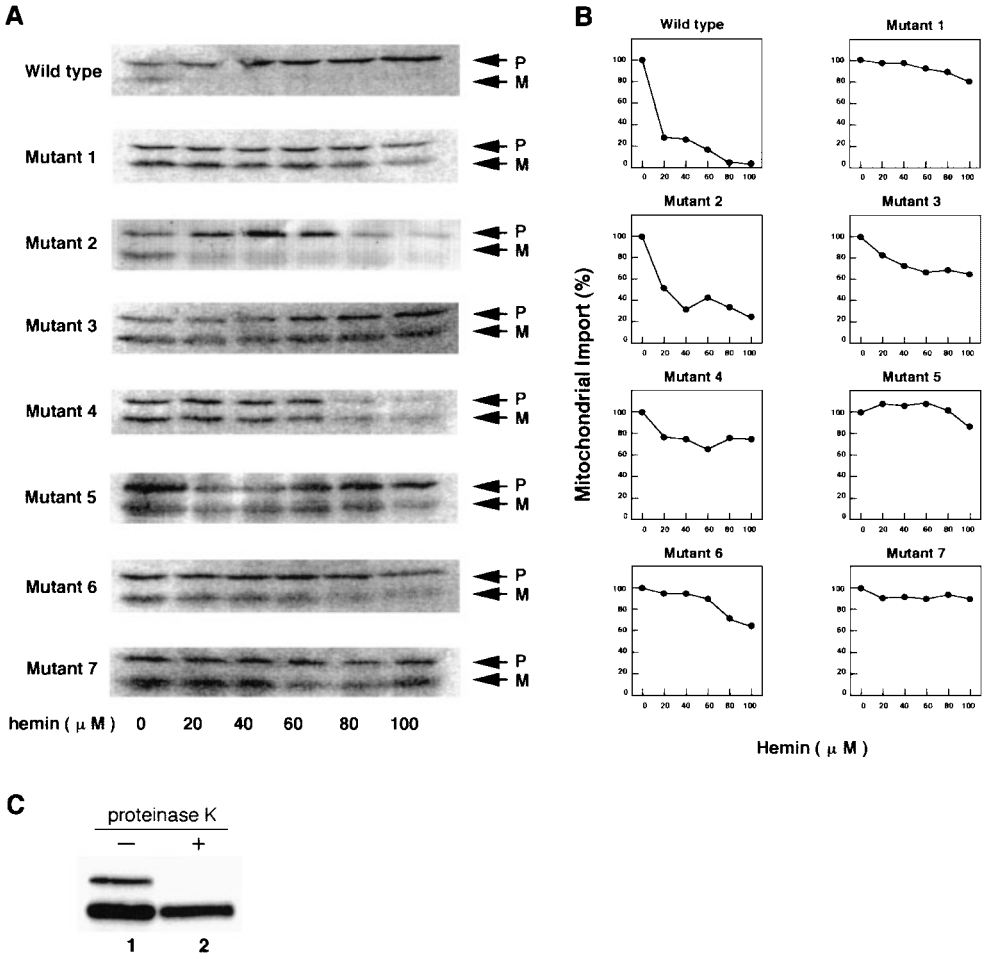

Fig. 2. **Mitochondrial import of wild-type and mutant ALAS1.** A: The wild-type and mutant rat ALAS1 proteins were expressed in quail fibroblast cell line QT6 with increasing concentrations (μM) of hemin, as indicated. SDS-PAGE was performed in 12% gels. After transfer to an Immobilon P membrane, proteins were immunoblotted with an anti-ALAS1 antibody. P and M denote the precursor and mature forms of ALAS1, respectively. The intensities of bands were calculated with NIH image software. B: Mitochondrial import was calculated as follows: ALAS imported into mitochondria (M) was divided by total ALAS (P + M). Values were expressed relative to the value with no added hemin, which was set at 100%, and were plotted against the hemin concentration. C: Mitochondria were isolated from QT6 cells and treated with proteinase K before SDS-PAGE (lane 2). Non-treated mitochondria were electrophoresed as well (lane 1).

were harvested. Mitochondria were isolated using a Mitochondria Isolation Kit (Sigma) employing the method recommended by the manufacturer. Cells were homogenized in Extraction Buffer A with a Potter-Elvehjem homogenizer and then centrifuged at 600 × *g* for 5 min. The supernatant was centrifuged at 11,000 × *g* for 10 min. The pellet was suspended in Extraction Buffer A. A part of the suspension was treated with proteinase K (200 μg/ml) for 30 min at 0°C. Then, the reaction was stopped by the adding phenylmethylsulfonylfluoride to the concentration of 10 mM.

Anti-ALAS1 and Anti-ALAS2 Antibodies—To obtain an anti-ALAS1 antibody, a synthetic peptide representing the C-terminal region of rat ALAS1 (amino acids 627–642, EKAYFSGMSKMVSAQA) was conjugated to bovine serum albumin and used to immunize rabbits. The rabbit serum of was subsequently affinity-purified on a synthetic peptide-ovalbumin–coupled resin.

To obtain an anti-ALAS2 antibody, a synthetic peptide representing the C-terminal region of rat ALAS2 (amino acids 568–587, SEWERSYFGNMGPQYVTTYA) was conjugated to keyhole limpet hemocyanine and used to immunize rabbits, followed by affinity-purification as above.

Detection of ALAS1 and ALAS2—Harvested cells were mixed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, heat-treated at 100°C for 3 min, and then centrifuged at 15,000 rpm for 10 min. The supernatant was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). The membranes were blocked overnight with 5% skimmed milk in TTBS and then treated with the anti-ALAS1 or anti-ALAS2 antibodies for 2 h. After extensive washing with TTBS, the blots were further probed with a secondary antibody conjugated to horseradish peroxidase against rabbit IgG. ALAS1 and ALAS2 were visualized using a KONIKA immunostaining kit (Seikagaku Kougyou, Japan), the intensity of the bands being measured using NIH Image software.

RESULTS AND DISCUSSION

ALAS1 has three HRMs, the sequences of which are shown in Fig. 1. We introduced mutations into the HRMs by converting cysteines to serines in all possible combinations (Fig. 1). Cysteines in HRMs are essential for their activity: heme has been shown to bind in a stoichiometric manner to cysteines in synthetic peptides containing an HRM sequence (*11*). Figure 1 shows the wild-type and mutated cDNAs of ALAS1, which were subcloned into pCAGGS, a strong eukaryotic expression vector containing the CMV promoter (*18*). After transfection of these cDNAs, cell lysates were subjected to SDS-PAGE and Western blot analysis.

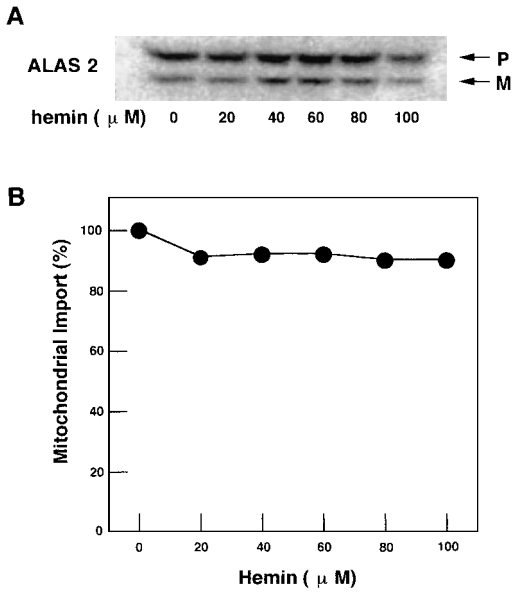

Fig. 3. **Mitochondrial import of ALAS2.** A: Rat ALAS2 was expressed in quail fibroblast cell line QT6 with increasing concentrations (μM) of hemin, as indicated. SDS-PAGE was performed in 12% gels. After transfer to an Immobilon P membrane, proteins were immunoblotted with an anti-ALAS2 antibody. P and M denote the precursor and mature forms of ALAS2. The intensities of bands were calculated with NIH image software. B: Mitochondrial import was calculated as described in the legend to Fig. 2.

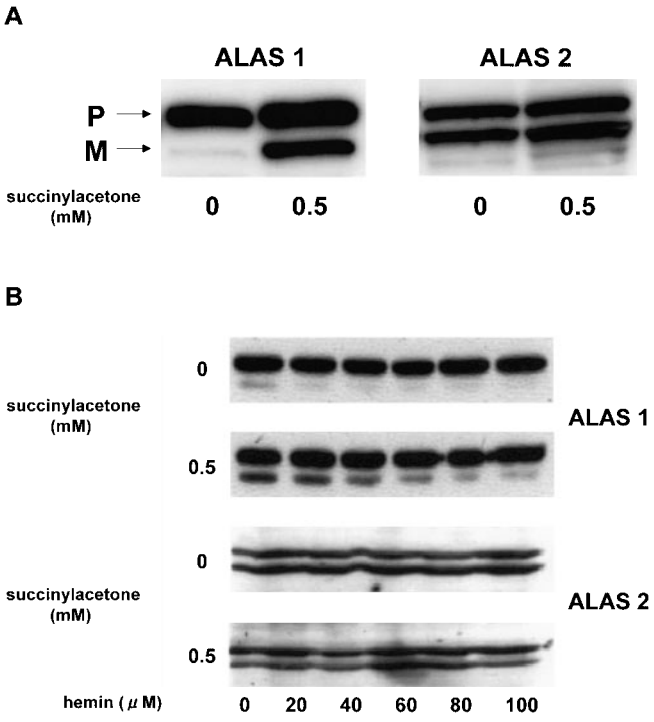

Fig. 4. **Mitochondrial import of ALAS1 and ALAS2 in the presence of succinylacetone.** ALAS1 and ALAS2 were expressed in QT6 cells and then lysates were examined for mitochondrial import.

The anti-ALAS1 antibody detected two bands, the migration of which corresponded to the molecular weights of the precursor and mature forms of ALAS1 (71 kDa and 65 kDa, respectively). To confirm that the lower molecular weight form of the detected material is the mature enzyme inside mitochondria, and the higher molecular weight one a precursor form, we isolated mitochondria from ALAS-transfected QT6 cells, followed by treatment with proteinase K. The results shown in Fig. 2 indicate that the higher molecular weight band disappeared on proteinase K treatment, but the lower molecular weight one did not. Only a small fraction of wild-type ALAS1 was imported into mitochondria (Fig. 2). When the cysteines in all three HRMs were changed to serines (mutant 7), the mitochondrial import of ALAS1 greatly increased (Fig. 2), suggesting that the heme inhibition of the mitochondrial import of ALAS1 is dependent on the presence of an HRM. In mutants 1–3, the cysteine residues in HRM1, HRM2 and HRM3, respectively, were changed to serine ones (Fig. 1). Heme did not inhibit the import of mutant 1 into mitochondria and had only a small effect on the mitochondrial import of mutant 3. By contrast, a low concentration of heme inhibited the import of mutant 2 (Fig. 2). These results indicate that HRM1 is involved in the heme-mediated inhibition of the mitochondrial import of rat ALAS1, whereas HRM2 is not. In addition, HRM3 contributes to the heme-mediated inhibition of ALAS1 transport.

These results were further confirmed by examining the relationship between the HRMs and the effect of heme on mitochondrial import using mutants 4, 5 and 6, in which one of the three HRMs was intact and the other two were mutated (Fig. 1). Hemin inhibited the mitochondrial import of mutant 4 (HRM1 is intact) and mutant 6 (HRM3 is intact), but not that of mutant 5 (HRM2 is intact). In conclusion, HRM1 and HRM3, workings together, are required for heme to be able to inhibit the mitochondrial import of rat ALAS1, whereas HRM2 has no effect on this inhibition. The precursor band intensity of mutants 2 and 4 seem to decrease in the presence of a high concentration of hemin (Fig. 2). However, considering the fact that the presence of hemin did not affect the expression of other mutants having similar amino acid mutations, it is more likely that the change in the intensities of the bands is due to the efficiency of transfection rather than the stability of the precursor proteins.

The HRM motif consists of five amino acids, among which the second cysteine residue and the third proline residue are completely conserved, while the other amino acids are not. In this context, HRM2 differs from other HRMs in that the first amino acid is basic in HRM1 and HRM3, but is neutral in HRM2, and that the fourth and fifth amino acids are phenylalanine and leucine in the former, but are lysine and methionine in HRM2 (Fig. 1). It is possible that these differences in amino acid sequence reflect the activity of the HRMs, although other ALAS1 mutants will have to be examined to confirm this hypothesis.

Rat ALAS2 cDNA was cloned into pCAGGS and the effect of heme on mitochondrial import was examined under the same conditions as for ALAS1. The anti-ALAS2 antibody detected two bands, the migration of which corresponded to the molecular weights of the precursor and mature forms of ALAS2 (65 kDa and 60 kDa,

respectively). We found that heme did not affect the mitochondrial import of ALAS2 (Fig. 3). The mitochondrial import of ALAS2 was not affected by a wide range of heme concentrations *in vivo*. By contrast, Lathrop and Timko, using mouse ALAS2, showed that the mitochondrial import of ALAS2 was inhibited by hemin. Furthermore, they showed that this inhibition was mediated by the HRM in the presequence and that just one HRM was enough to cause the inhibition (*15*). Although their results seem to contradict our study, Lathrop and Timko used *in vitro* systems, whereas here we used an *in vivo* system. Thus, the different results may be due to the experimental conditions. The heme inhibition of the transport of mouse ALAS2 may also differ from that of rat ALAS2. Another possibility is that the positions of the HRMs in the protein may affect the inhibitory activity of heme. It should also be pointed out that the hemin concentration in our case is the concentration in the growth medium, not in the cell, and as such is considered to be lower than the concentration used in the *in vitro* experiments of Lathrop and Timko. If the heme concentration in the cell were increased to the levels used under *in vitro* conditions, then inhibition of ALAS2 transport might occur. Recently, evidence that heme actually binds to ALAS2 HRM was presented by Goodfellow *et al*. They synthesized the presequence of ALAS2 and a 26 amino acid N-terminal fragment of the presequence. Titration and ^{1}H NMR studies showed that heme interacts with both HRM 1 and HRM 2 in ALAS2 (*19*).

We wanted to confirm independently that heme inhibits the mitochondrial import of ALAS1 but has no effect on the mitochondrial import of ALAS2. It seemed likely that the concentration of heme would be higher in cells transfected with ALAS cDNA than in untransfected cells, and this might lower the mitochondrial import of ALAS. Therefore, we examined mitochondrial import under conditions in which succinylacetone, an inhibitor of 5-aminolevulinate dehydratase, was added to the medium to lower the endogenous heme concentration by reducing the amount of heme precursor. As expected, the mitochondrial import of wild-type ALAS1 was higher in the presence of succinylacetone than in its absence (Fig. 4). This strongly suggests that the mitochondrial import of wild-type ALAS1 is inhibited by very low concentrations of heme. In contrast, succinylacetone had no effect on the mitochondrial import of ALAS2.

It seems reasonable that the mitochondrial import of ALAS2 is not inhibited by heme in erythroid cells because, during erythroid differentiation, hemoglobin synthesis is in progress and a large amount of heme must be synthesized; in turn, the enzymatic activity of ALAS in erythroid cells must be high enough to fulfill the substrate needs for heme synthesis and ultimately hemoglobin synthesis. In the case of ALAS1, whose function is to maintain the basal level of heme needed for cell survival, the local levels of heme should be maintained within in a narrow concentration range. In the case of ALAS2, and especially during the period of erythroid development, a large amount of heme must be synthesized to make hemoglobin (*20*), and thus the concentration of heme need not be controlled so finely. In addition, in developing erythroid cells, the concentration of free heme may not be high enough to inhibit the mitochondrial import of ALAS because most heme molecules are bound to globin.

Although both ALAS1 and ALAS2 possess three HRMs, the inhibition of their mitochondrial import by heme differs, as described above. The exact amino acid sequences of the HRMs, the positions of the HRMs in the primary structure, as well as the distances between the HRMs may be factors that affect the activity of the HRMs through which heme mediates its inhibitory effects on the transport of ALAS. These factors remain to be clarified in the future.

Using partially purified ALAS1, Scholnick *et al*. showed that heme directly inhibits the activity of ALAS1. We purified ALAS1 from rat liver and ALAS2 from rat reticulocytes, and compared the effect of heme on the activities of ALAS1 and ALAS2. The two enzymes were inhibited by heme to the same extent (Munakata *et al*., unpublished data). Particularly noteworthy is that proteolytic enzyme papain was used during purification, and that the purified enzymes do not contain HRMs. This observation suggests the possibility of the involvement of a heme binding motif other than HRM in the allosteric inhibition of ALAS activity by heme.

ALAS1 and ALAS2 are probably derived from a common ancestor protein (*21*), and it is quite likely that during evolution the HRMs in these enzymes have changed to fulfill the requirements for heme synthesis in the different situations in which the two enzymes function. Considering the biological roles of the two isoforms of ALAS, it seems likely that the activity of ALAS1 is regulated through the inhibition of mitochondrial import by the intracellular concentration of heme, whereas the activity of ALAS2 is mainly regulated translationally by the intracellular concentration of iron.

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