The Role of a FAD Cofactor in the Regulation of Acetohydroxyacid Synthase by Redox Signaling Molecules*

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Acetohydroxyacid synthase (AHAS) catalyzes the first step of branched-chain amino acid (BCAA) biosynthesis, a pathway essential to the lifecycle of plants and microorganisms. This enzyme is of high interest because its inhibition is at the base of the exceptional potency of herbicides and potentially a target for the discovery of new antimicrobial drugs. The enzyme has conserved attributes from its predicted ancestor, pyruvate oxidase, such as a ubiquinone-binding site and the requirement for FAD as cofactor. Here, we show that these requirements are linked to the regulation of AHAS in relationship to its anabolic function. Using various soluble quinone derivatives (e.g. ubiquinones), we reveal a new path of down-regulation of AHAS activity involving inhibition by oxidized redox-signaling molecules. The inhibition process relies on two factors specific to AHAS: (i) the requirement of a reduced FAD cofactor for the enzyme to be active and (ii) a characteristic slow rate of FAD reduction by the pyruvate oxidase side reaction of the enzyme. The mechanism of inhibition involves the oxidation of the FAD cofactor, leading to a time-dependent inhibition of AHAS correlated with the slow process of FAD re-reduction. The existence and conservation of such a complex mechanism suggests that the redox level of the environment regulates the BCAA biosynthesis pathway. This mode of regulation appears to be the foundation of the inhibitory activity of many of the commercial herbicides that target AHAS.

Results and Discussion

AHAS activity is characterized by a temperature-dependent lag phase requiring incubation with substrate before reaching maximal activity (5). Monitoring the ScAHAS activity at 333 nm (6) and the absorbance of oxidized FAD (FADox) at 450 nm under identical assay conditions with a saturating concentration of pyruvate revealed that the concentration of enzyme-bound FADox decreases during the lag phase, FAD being fully reduced when the enzyme reaches steady-state activity (Fig. 2, A and B). The reduction of ScAHAS-bound FADox occurs in two phases. In the first phase, the reduction is rapid and involves around 25–30% of the total FAD. This phase could be attributed to the pre-existence of an enzyme-hydroxyethyl-ThDP complex allowing rapid reduction of FADox through a long-range electron transfer (7). In the second phase, the FAD reduction rate is lower and follows an exponential decay. To assess the correlation between FAD reduction and enzyme activation during phase II (Fig. 2C), two equations were derived (see “Experimental Procedures” for equation derivation).

\[
[P] = k_{cat}\left([E_{tot}]t + \left([E_{tot}] - [E_0]e^{k_{obs(sat)}\frac{1}{k_{obs(dct)}}}\right)\right) \tag{1}
\]

Equation 1, which represents the variation of the product concentration \([P]\) as a function of time, where \([E_{tot}]\) represents the total concentration of enzyme, \([E_0]\) the concentration of enzyme that is initially activated during the rapid first phase of
activation (Phase I, Fig. 2, A and C), $k_{\text{cat}}$ the catalytic constant, and $k_{\text{obs(act)}}$ the “observed” first-order rate of AHAS activation. The equation was used to fit the experimental curves, giving an average $k_{\text{obs(act)}}$ value of 0.122 ± 0.016 min$^{-1}$ (S.E. of the mean of five measurements).

$$A_{450} = \xi_1[FAD_{\text{tot}}] + (\xi_2 - \xi_1)([FAD_{\text{tot}}] - [FAD_0])(e^{-k_{\text{obs(act)}}t})$$

(Eq. 2)

Equation 2, which represents the variation of the absorbance of FAD at 450 nm as a function of time, where [FAD$_{\text{tot}}$] represents the total concentration of enzyme-bound FAD, [FAD$_0$] the concentration of FAD that is initially reduced during the rapid first phase of activation, $\xi_1$ and $\xi_2$, the (unknown) molar absorption coefficients at 450 nm of enzyme-bound reduced FAD (FAD$^\text{red}$) and FAD$^\text{ox}$, and $k_{\text{obs(red)}}$ the observed first-order rate constant of FAD$^\text{ox}$ reduction. The equation was used to fit the experimental curves, yielding an average $k_{\text{obs(red)}}$ value of 0.120 ± 0.013 min$^{-1}$ (S.E. of the mean of five measurements). Remarkably, the value of $k_{\text{obs(red)}}$ correlates very well with the value of $k_{\text{obs(act)}}$. The relationship between the enzyme activity and the redox status of enzyme-bound FAD is further demonstrated by the fact that addition of pyruvate to the reaction cuvette at the end of the first reaction where FAD has been fully reduced leads to a reaction velocity characterized by the absence of a lag phase (Fig. 2A). A good correlation between the rates of FAD reduction and enzyme activation was also observed for AHAS from Mycobacterium tuberculosis (MtAHAS, catalytic subunit ilvB1) (Fig. 3), suggesting that this feature is generic in AHASs and that a reduced FAD cofactor is imperative for AHAS activity.

Considering the absorbance profile of reduced FAD is perturbed by the presence of pyruvate/acetolactate (Fig. 2B), it was not possible to determine which form of reduced FAD (FAD fully reduced or the semiquinone form) is present during steady-state catalysis. To eliminate the contributions of pyruvate/acetolactate in the UV-visible spectrum, an experiment was undertaken where the activated enzyme was rapidly purified on a desalting column. However, the result was not a spec-
It is known for example that conditions because the half-life of AHAS could be quite short. This obstacle for achieving full activity under physiological conditions is the rate of FAD reduction (and enzyme activation). Only the initial part of the curves were fitted, where the redox state of FAD is affected by the environment within the enzyme.

Tittmann et al. (8) have demonstrated that the cofactor, FAD, can be reduced by the POX side activity of AHAS and in light of our results, it is evident that this reaction is not productive but serves to activate the enzyme. Importantly, Tittmann et al. (8) also found that FADred is very unreactive toward molecular oxygen, and this feature is indeed crucial to maintain the enzyme active in aerobic conditions. Consistent with FAD being catalytically reduced by pyruvate, the variation of \( k_{\text{obs}}(\text{act}) \) as a function of the concentration of pyruvate follows Michaelis-Menten kinetics with \( K_m \) values of 24 and 90 mM for ScAHAS and MtAHAS, respectively (Fig. 4). The plateau of the curves corresponds to the catalytic rate constant \( (k_c) \) of AHAS activation (POX activity), which has at 30 °C a value of 0.83 and 0.28 min⁻¹ for ScAHAS and MtAHAS, respectively. These results imply that at physiological concentrations of pyruvate, the rate of FAD reduction (and enzyme activation) is expected to be slow (e.g. if [pyruvate] ≤ 1 mM, \( k_{\text{obs}}(\text{act}) \) ≤ 0.003/0.04 min⁻¹ (corresponding to a half-time of activation ≥230/17 min) for MtAHAS/ScAHAS at 30 °C). The seemingly low activation rate, especially for MtAHAS, could appear to be an obstacle for achieving full activity under physiological conditions because the half-life of AHAS could be quite short. It is known for example that ScAHAS has a half-life of 8.5 h (yeastmine.yeastgenome.org), but there is no equivalent data available for the Mt enzyme. There are also a number of other factors that could come into play in achieving high enzymatic activity within cell. These include the concentration of pyruvate, rate of protein synthesis, and the availability of the regulatory subunit. Further studies need to be performed to dissect the role of each factor in activating AHAS under the physiological conditions of the cell.

To investigate the role of the FAD cofactor in AHAS inhibition by ubiquinones, 50 \( \mu \)M Q1 was reacted with MtAHAS and three events were monitored: (i) the activity of MtAHAS at 333 nm, (ii) the redox state of FAD cofactor at 450 nm, and (iii) the redox state of Q1 at 405 nm (spectral peak, Fig. 5A). MtAHAS inhibition by Q1 appears to be time-dependent (as reported for the inhibition of ALS1–3 by Q0 (3)) and in correlation with the progressive oxidation of the FAD cofactor (Fig. 6A). The inhibition by Q1 is also associated with the reduction of Q1 (Fig. 6B), inferring that a redox reaction occurs between Q1 and FAD. When Q1 is fully reduced by addition of 1 mM dithiothreitol (DTT), the enzyme recovers activity meaning that Q1 has the ability to inhibit AHAS activity only in its oxidized form. The recovery of the enzyme activity is slow and linked to the re-reduction of FAD (Fig. 6A). These results indicate that FAD oxidation by Q1 is the main driver of the inhibition process.

For ALS1–3, the initial inhibition by Q1 is weak and non-competitive (3). Here, the inhibition curves (Fig. 6, A and B) show that the initial inhibition of MtAHAS by 50 \( \mu \)M of Q1 is small, implying a weak affinity. The initial inhibition of 1.7 \( \mu \)M MtAHAS was determined for different concentrations of Q1.
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The influence of the pyruvate concentration on the inhibition of AHAS by Q₁ is a feature intimating that regulation of AHAS by the redox level of the environment is of physiological relevance. Indeed, a low redox level in the mitochondria/cell correlates with a low production of energy (Fig. 1). In this situation, if the concentration of pyruvate (precursor molecule for energy production) is low, the inhibition of the anabolic BCAA synthetic pathway is logical. However, a high concentration of pyruvate has the potential to rapidly increase the redox level and energy production required for an anabolic process (Fig. 1), and consistently, a reduction of the inhibitory potency of Q₁ is observed in our experiments. Q₀ has been shown to inhibit ALS₁–₃ and here we show that it also inhibits MtAHAS and Arabidopsis thaliana AHAS (AtAHAS) (Fig. 8, A and B), through a mechanism apparently similar to the one involved in inhibition by Q₁.

Here we also show that other aromatic molecules, different from ubiquinone, can inhibit AHAS. Menadione, an oxidized quinone derivative that serves as a redox mediator in Klebsiella pneumoniae (9), inhibits MtAHAS in a similar fashion to Q₀ and Q₁ (Fig. 9). These results suggest that the regulation by oxidized soluble quinones is generic for AHAS from all organisms (bacteria, fungi, plants) and that different molecules can serve as redox signaling molecules, presumably specific to each kind of organism.

Schloss (3) has shown that Q₀ and the commercial AHAS inhibiting herbicides compete for the same binding site. Here, docking studies confirm that Q₁ fits neatly into the herbicide binding pocket (Fig. 10). The model suggests that electrons that are transferred from reduced FAD to Q₁ could pass through the aromatic ring of Phe-201. The ring is 3.51 Å from the C7 carbon atom of FAD and 3.56 Å from the aromatic ring of Q₁, distances that can be traversed by the electrons (10). Phe-201 can also serve as a relay for electrons transferred from hydroxyethyl-ThDP to FADₐox during the POX side reaction that activates the enzyme. This arrangement resembles that observed in Lactobacillus plantarum POX, where the transfer of two electrons from hydroxyethyl-ThDP to FADₐox occurs through the aromatic ring of a phenylalanine residue (Phe-121 or Phe-479) (11).

As the soluble quinone derivatives and herbicides bind to the same pocket in AHAS, a comparison of their mode of inhibition is worthy of discussion. The major difference is that the herbicides have a high affinity (i.e. sulfonylureas have Kᵢ values in the low nM range), whereas the quinones have a much weaker affinity (>100 μM, Ref. 3 and this study) for AHAS. The low affinity of quinones may reflect the need for AHAS to be able to accommodate different types of quinones and also to prevent the inhibition of AHAS by standard concentrations of quinones at normal physiological conditions. In contrast, herbicides have a high affinity for AHAS because they have been designed to maximally inhibit under all conditions. Another important aspect, which is different between the herbicides and quinones, is that herbicides are apparently not altered by interaction with AHAS, whereas the quinones are subject to modification (reduction by the enzyme-bound FADₐred) that renders the molecule ineffective for further inhibition. This is a crucial aspect in the regulation of AHAS activity by quinones as this feature...
would prevent the enzyme from being affected by small decreases of redox level that are not relevant physiologically. Indeed, the quinone reduction activity would allow AHAS to quickly re-establish normal redox conditions in the cell, restoring normal AHAS activity.

![Figure 6. Inhibition of MtAHAS by Q1.](image)

FIGURE 6. Inhibition of MtAHAS by Q1. A, progress curves for the inhibition of MtAHAS by Q1 monitored at 333 (pyruvate absorbance, upper curve) and 450 nm (intrinsic FAD absorbance, lower curve) under identical assay conditions. 7 μM MtAHAS was incubated with 100 μM pyruvate in standard buffer in the absence of free FAD at 35 °C. After reaching full activation (17 min), the inhibition reaction was initiated by adding 50 μM Q1 in the sample cell. After 7 min of incubation with Q1 associated with the oxidation of the FAD cofactor an the inhibition of MtAHAS activity by 80%, 1 mM DTT was added in the sample cell to reduce the remaining Q1, triggering the re-activation of the enzyme and re-reduction of FAD. Notably the progress curve at 450 nm not only monitors FAD reduction/oxidation, but also Q1 concentration (Fig. 5A), which is reflected by the absorbance jumps observed upon injection of Q1 and DTT (arrows). B, progress curves for the inhibition of MtAHAS by Q1 monitored at 333 (upper curve) and 405 nm (spectral peak of Q1, Fig. 5A) lower curve under identical assay conditions. 1.7 μM MtAHAS was incubated with 100 μM pyruvate in standard buffer in the absence of free FAD at 30 °C. After activation of MtAHAS (8 min), the inhibition reaction was initiated by adding 50 μM Q1 in the sample cell. After 11 min of incubation associated with the reduction of Q1, the inhibition of MtAHAS activity reached 74%. 1 mM DTT was then added in the sample cell to reduce the remaining Q1, allowing the re-activation of the enzyme. Due to the low concentration of MtAHAS in this experiment, the progress curve at 405 nm monitoring Q1 reduction was not significantly affected by the reduction/oxidation of the FAD cofactor. C, initial inhibition of MtAHAS as a function of Q1 concentration. 1.7 μM activated MtAHAS was incubated with different concentrations of Q1 (25–500 μM) and a calculation of the initial inhibition was performed, as illustrated in the inset where curves a, b, and c correspond to inhibition by 500, 50, and 0 μM Q1, respectively. The dotted lines represent linear regressions made with data collected during the initial 50 s of inhibition. The measurement of the initial inhibition was feasible only to Q1 concentrations ≤ 500 μM (“Experimental Procedures”). The data were fitted using Equation 3 yielding an estimated $K_i$ value of 240 μM. D, scheme representing the mechanism of inhibition of AHAS by Q1. $E_{ox}/E_{red}$ represents the enzyme with oxidized/reduced FAD cofactor, pY the pyruvate molecule, $Q_{1_{red}}$ the reduced form of Q1, $k_{act}$ the rate constant of enzyme activation, and $k_{act}$ the rate constant of FAD oxidation by Q1.

Besides these differences, there is a commonality between quinones and the commercial herbicides in that their mode of inhibition is time-dependent and linked to oxidation of the FAD$_{red}$ (7). However, the mechanisms leading to this common phenomenon are unrelated. As shown here, the quinones directly oxidize FAD$_{red}$, whereas the herbicides stabilize a ThDP-peracetate intermediate derived from the inherent oxygenase side reaction of AHAS,4 ultimately leading to the release of reactive oxygen species (peracetate, singlet oxygen) that oxidizes FAD (7). Thus, the high potency of herbicides appears to be the consequence of a combination of exceptional circumstances, linked to the multiple reactions that can occur in the AHAS active and herbicide/quinone binding sites.

Our results explain two subjects of conjecture, i.e. why AHAS has a highly conserved “ancestral” ubiquinone-binding site and also why it has retained its requirement for FAD (3, 4). It is because both features are an integral part of a mechanism of critical importance for the regulation of BCAA synthesis. In light of this research, it appears that this novel regulation pathway is at the core of the inhibitory activity of many of the commercial herbicides that target AHAS.

4 T. Lonhienne, M. D. Garcia, and L. W. Guddat, unpublished data.
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FIGURE 8. The inhibition of MtAHAS and AtAHAS by Q0. Progress curves for the inhibition of MtAHAS (A) and AtAHAS (B) by Q0, monitored at 333 nm (upper curve, left y axis) and 408 nm (spectral peak of Q0, Fig. 5B, lower curve, right y axis) under identical assay conditions. A. 2.5 μM MtAHAS was incubated with 50 mM pyruvate in standard buffer at 30 °C. After activation of the enzyme (10 min), the inhibition reaction was initiated by adding 40 μM Q0 in the sample cell. The progress curves show that the inhibition of MtAHAS activity correlates with the reduction of Q0. The reactivation of the enzyme occurs when the majority of Q0 is reduced, in a time-dependent fashion consistent with the re-reduction of FAD by the POX side activity of the enzyme. B. 0.8 μM AtAHAS was incubated with 10 mM pyruvate in standard buffer at 30 °C. After reaching maximum velocity (12 min), 80 μM Q0 was added to the sample cell. After 12 min of incubation associated with the reduction of Q0 and an inhibition of AtAHAS activity by 78%, 1 mM DTT was added to reduce the remaining Q0, resulting in the reactivation of AtAHAS.

FIGURE 9. The inhibition of MtAHAS by menadione. Progress curve of the inhibition of 250 nM MtAHAS with 20 μM menadione, using a colorimetric assay (“Experimental Procedures”). After 13 min of incubation in standard buffer at 30 °C allowing the reaction to reach maximum velocity, 20 μM menadione was added to the reaction cell resulting in almost full inhibition of MtAHAS activity. After 12 min of incubation with menadione, 1 mM DTT was added in the sample cell to reduce the remaining menadione, resulting in the reactivation of MtAHAS.

Experimental Procedures

Protein Preparation

The expression and purification of MtAHAS and ScAHAS was carried out in two steps as described previously (IMAC and gel filtration) (12), however, with a modification: the gel-filtration purification step was performed on a Sephacryl S200 size exclusion FPLC column and Mt- or ScAHAS were eluted in buffer that contained 0.2 M potassium phosphate, pH 7.2, and 10 μM FAD. Using a Millipore Centricon with a 30-kDa cut-off, Mt- or ScAHAS was then concentrated to 35–64 mg/ml and aliquots were snap cooled at ~80 °C. This process led to highly concentrated enzyme (480–870 μM) carrying the cofactor FAD, and giving an enzyme solution in which the ratio of enzyme-bound FAD versus free FAD is ~40 in favor of the enzyme-bound FAD.

The expression and purification of AtAHAS was carried as described previously (13). Enzyme concentrations were determined by using a Direct Detect spectrophotometer (Millipore).

Enzyme Assays

Enzyme assays were performed using a continuous spectrophotometric method measuring the disappearance of pyruvate (6) or a colorimetric method (14). The standard buffer contained 200 mM potassium phosphate, pH 7.2, 1 mM ThDP, 10 mM MgCl2, and 10 μM FAD. The concentration of pyruvate was variable and described in the text, under “Experimental Procedures,” or in the figure legends.

Time Course Study of ScAHAS Lag Phase (Fig. 2A)

ScAHAS activity was measured at 333 nm by the continuous spectrophotometric method, whereas reduction-oxidation of enzyme-bound FAD was monitored at 450 nm (intrinsic absorbance of FAD), under identical assay conditions. The assays were performed in standard buffer in the absence of free FAD to avoid interference with the enzyme-bound FAD. The first reaction involved the incubation of 9.5 μM ScAHAS with 100 mM pyruvate at 15 °C (total volume of 200 μl) for 36 min until full depletion of pyruvate occurred. A low temperature of 15 °C was chosen to increase the time of the lag phase and therefore to increase the accuracy of its analysis. The second reaction (Fig. 2A, arrow) was initiated by addition of 7 μl of a 3 M pyruvate stock solution (pyruvate dissolved in 200 mM potassium phosphate, pH 7.2) in the sample cell. The addition of a high pyruvate concentration ensured that the extra volume did not significantly alter the concentration of the enzyme in the sample cell.

Desalting of ScAHAS (Fig. 2D)

Two samples (samples 1 and 2) were analyzed. Sample 1 consisted of a 1-ml solution of 41 μM ScAHAS in standard buffer that did not include free FAD and pyruvate. Sample 2 consisted of a 1-ml solution of 41 μM ScAHAS (in standard buffer exempt of free FAD) incubated with 1.4 mM pyruvate for 30 min at 15 °C. Note that the pH (7.2) was not affected by the high concentration of pyruvate. Spectrophotometric monitoring of the reaction occurring in sample 2 confirmed that after 30 min of reaction, ScAHAS was in steady-state activity and that the sample still contained significant concentration of pyruvate.

Each sample was desalted on a PD-10 column equilibrated at 5 °C with a buffer (50 mM KP, 1 mM ThDP, 10 mM Mg) de-oxygenated by N2 bubbling to minimize FAD re-oxidation during the desalting process. For sample 1, which was yellow (color of oxidized FAD), 10 of the most intensely yellow drops were col-
Time Course Study of MtAHAS Lag Phase (Fig. 3)

21.5 μM MtAHAS was incubated with 300 mM pyruvate at 30 °C. MtAHAS activity was measured by the continuous spectrophotometric method at 360 nm instead of the typical 333 nm to avoid absorbance saturation linked to the high concentration of pyruvate used in the assays.

Equations for AHAS Activation and Enzyme-bound FAD Reduction (Figs. 2C and 3B)

The equations were derived as follows. For the rate of AHAS activation,

\[
\frac{d[E_{act}]}{dt} = k_{obs(act)}[E_{nact}] = k_{obs(act)}([E_{tot}] - [E_{act}]) \quad \text{(Eq. 4)}
\]

where \(k_{obs(act)}\) represents the first-order rate of AHAS activation observed at a pyruvate concentration specific to a particular experiment, and where \([E_{tot}]\), \([E_{act}]\), and \([E_{nact}]\) represent the concentration of total enzyme, enzyme activated, and enzyme inactivated, respectively.

Integration of Equation 4 gives,

\[
[E_{act}] = [E_{tot}] - ([E_{tot}] - [E_0]) e^{-k_{obs(act)}t} \quad \text{(Eq. 5)}
\]

where \([E_0]\) represents the concentration of ScAHAS that is initially activated during the rapid first phase of activation (Fig. 2).

Note that for MtAHAS, \([E_0] = 0\) as there is only one phase (Fig. 3).

At saturating substrate conditions for the AHAS reaction,

\[
\frac{d[P]}{dt} = k_{cat}[E_{act}] = k_{cat}([E_{tot}] - ([E_{tot}] - [E_0]) e^{-k_{obs(act)}t}) \quad \text{(Eq. 6)}
\]

where \([P]\) represents the product concentration.

Integration of Equation 6 gives Equation 7.

\[
[P] = k_{cat}([E_{tot}] t + ([E_{tot}] - [E_0]) \frac{e^{-k_{obs(act)}t} - 1}{k_{obs(act)}}) \quad \text{(Eq. 7)}
\]

Taking into account that the product (acetolactate) is the result of the condensation of two pyruvate molecules and that the extinction coefficient of pyruvate is 17.5 M⁻¹ cm⁻¹ at 333 nm, Equation 7 can be transformed as follows,

\[
A_{333} = -0.000035 k_{cat}([E_{tot}] t + ([E_{tot}] - [E_0]) \frac{e^{-k_{obs(act)}t} - 1}{k_{obs(act)}}) + C \quad \text{(Eq. 8)}
\]

where \(A_{333}\) represents the absorbance of pyruvate and where the concentration of enzyme is given in micromolar.

The raw data were fitted with Equation 8 using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). The parameter \(E_{tot}\) was known and fixed. \(k_{cat}\), \(E_0\), and \(k_{obs(act)}\) were variable parameters.

For the first-order rate of FAD reduction,

\[
\frac{d[FAD_{red}]}{dt} = k_{obs(red)}[FAD_{red}] = k_{obs(red)}([FAD_{tot}] - [FAD_{red}]) \quad \text{(Eq. 9)}
\]

where \(k_{obs(red)}\) represent the observed first-order rate constant of FAD reduction and \([FAD_{tot}]\), \([FAD_{red}]\), and \([FAD_{tot}]\) represent the concentration of total enzyme-bound.
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FAD, enzyme-bound reduced FAD, enzyme-bound oxidized FAD, respectively.

Integration of Equation 9 gives

\[
[FAD_{\text{red}}] = [FAD_{\text{tot}}] - ([FAD_{\text{tot}}] - [FAD_0])(e^{-k_{\text{obs(red)}}}) 
\] (Eq. 10)

where \([FAD_0]\) represents the concentration of FAD that is initially reduced during the rapid first phase of activation (Fig. 2).

[FAD_{\text{ox}}] is deduced from Equation 10.

\[
[FAD_{\text{ox}}] = [FAD_{\text{tot}}] - [FAD_{\text{red}}] = ([FAD_{\text{tot}}] - [FAD_0])(e^{-k_{\text{obs(red)}}}) 
\] (Eq. 11)

The total absorbance at 450 nm is represented by,

\[
A_{450} = \xi_1[FAD_{\text{red}}] + \xi_2[FAD_{\text{ox}}] 
\] (Eq. 12)

where \(\xi_1\) and \(\xi_2\) represent the (unknown) absorption coefficients at 450 nm of enzyme-bound reduced FAD and FAD ox.

Using Equations 10 and 11, Equation 12 was transformed to,

\[
A_{450} = \xi_1[FAD_{\text{tot}}] + (\xi_2 - \xi_1)([FAD_{\text{tot}}] - [FAD_0])(e^{-k_{\text{obs(red)}}}) 
\] (Eq. 13)

or

\[
A_{450} = C_1 + C_2(e^{-k_{\text{obs(red)}}}) 
\] (Eq. 14)

where \(C_1\) and \(C_2\) represent constants equal to \(\xi_1[FAD_{\text{tot}}]\) and \((\xi_2 - \xi_1)([FAD_{\text{tot}}] - [FAD_0])\), respectively.

The raw data were fitted with Equation 14 using GraphPad Prism 6.0. \(C_1\), \(C_2\), and \(k_{\text{obs(red)}}\) were variable parameters.

Calculation of the Michaelis-Menten Constant (\(K_m\)) of Pyruvate for the POX Side Reaction That Activates AHAS (Fig. 4)

The continuous spectrophotometric method was used for this experiment. The kinetic assays were performed at 30 °C, in the standard buffer containing different concentrations of pyruvate. At all concentrations of pyruvate tested (10–400 mM), the concentration of the enzyme was optimized to have less than 10–15% of the pyruvate consumed during the assay. At high concentrations of pyruvate (100–400 mM), the value of the absorption wavelength was increased (350, 360, or 370 nm) to avoid saturation. This does not affect the calculation of the rate of enzyme activation. The data were fitted to the Michaelis-Menten equation,

\[
k_{\text{obs(act)}} = k_{\text{act}} \frac{[\text{Pyr}]}{[\text{Pyr}]+K_m} 
\] (Eq. 15)

where \(k_{\text{obs(act)}}\) represents the observed rate of AHAS activation, \(k_{\text{act}}\) the catalytic rate constant of AHAS activation, and [pyr] the concentration of pyruvate.

Time Course Studies of MtAHAS Inhibition by \(Q_1\) (Fig. 6, A and B)

Monitoring of MtAHAS Activity (333 nm) and FAD Reduction/Oxidation (450 nm) in Identical Assays (Fig. 6A)—7 μM MtAHAS was incubated with 100 mM pyruvate in standard buffer in the absence of FAD at 25 °C (in a total volume of 198 μl in a Quartz cuvette) for 17 min to allow the full activation of the enzyme to occur. 2 μl of a solution of 5 mM \(Q_1\) (diluted in DMSO) was then added in the cuvette resulting in a concentration of 50 μM \(Q_1\). We verified that 1% DMSO did not significantly affect AHAS activity. After 7 min of incubation with \(Q_1\), 2 μl of a solution of 100 mM dithiothreitol (DTT) was added, reaching a final concentration of 1 mM able to fully reduce \(Q_1\) (Fig. 5A).

Monitoring of MtAHAS Activity (333 nm) and \(Q_1\) Reduction (405 nm) in Identical Assays (Fig. 6B)—The experiment was similar to the one mentioned above except for the concentration of MtAHAS (1.7 μM) and the temperature (30 °C).

Calculation of the Constant of Inhibition (\(K_i\)) of \(Q_1\), Inhibiting MtAHAS (Fig. 6C)

1.7 μM MtAHAS was incubated with 100 mM pyruvate in standard buffer at 30 °C, ensuring saturation by the substrate. After 8 min of incubation allowing almost full activation of the enzyme, various concentrations of \(Q_1\) (25 to 500 μM) were added and a linear regression calculation was performed with the data corresponding to the initial 50 s of the inhibition curve (see inset of Fig. 6C). The initial activity rates were plotted versus the concentration of \(Q_1\) and the curve was fitted with the equation for non-competitive inhibition.

\[
v_i = v_o/(1 + [I]/K_i) 
\] (Eq. 16)

Due to the rapid increase of inhibition with time, it was not possible to assess the initial inhibition at concentrations of \(Q_1\) higher than 500 μM.

Effect of the Pyruvate Concentration on the Inhibition of ScAHAS by \(Q_1\) (Fig. 7)

ScAHAS activity was measured using the colorimetric method. 60/30 nm ScAHAS was incubated with 4/100 mM pyruvate in standard buffer at 30 °C in a total volume of 2.46 ml. During the entire inhibition assay, 80-μl aliquots were taken every 2 min and added to an Eppendorf tube containing 14 μl of 50% H2SO4 (v/v) to stop the reaction and have a H2SO4 concentration adequate for the colorimetric assay (see below). After 11 min of incubation, 20 μl of a solution of 50 mM \(Q_1\) was added to the sample (remaining volume of 1.98 ml after 6 samplings of 80 μl), yielding a final concentration of 500 μM. Only for the sample that initially contained 4 mM pyruvate: after 16 min of incubation with \(Q_1\) (and a remaining sample volume of 1.36 ml), 7 μl of a solution of 1 mM DTT was added (final concentration of ~5 mM DTT) to reduce the remaining \(Q_1\). The colorimetric assay was performed as follows: the aliquots sampled during the reaction and treated with H2SO4 (see above) were incubated at 60 °C for 15 min (formation of acetoin). Then, 100 μl of creatine (0.5% w/v) and 100 μl of α-naphthol (diluted in 4 mM NaOH, 5% w/v) was added and samples were further incubated at 60 °C for 15 min (color development). 200 μl of the samples were transferred in a 96-well plate and absorbance was measured at 525 nm. Note that full catalytic conversion of 4 mM pyruvate to acetolactate leads to an absorbance ~ 10 AU in the same conditions, meaning that less than 20% pyruvate was consumed during the inhibition experiment (Fig. 7).

Inhibition of MtAHAS by Menadione (Fig. 9)

MtAHAS activity was measured using the colorimetric method. 250 nm MtAHAS was incubated with 5 mM pyruvate in
standard buffer at 30 °C in a total volume of 3 ml. During the entire inhibition assay, 80-μl aliquots were taken every minute and added to an Eppendorf tube containing 14 μl of 50% H2SO4 (v/v). After 12.5 min of incubation, 8 μl of a solution of 5 mM of menadione (dissolved in DMSO) was added to the sample (having a remaining volume of 1.96 ml after 13 samplings of 80 μl), yielding a final concentration of 20 μM. After 12 min of incubation with menadione (remaining volume of the sample was 1 ml), 10 μl of a solution of 100 mM DTT was added (final concentration of 1 mM DTT) to reduce the remaining menadione. The colorimetric assay was performed as described above.

Estimation of the First-order Rate Constant of FAD Oxidation by Q1

The slope of the linear part of the inhibition curve (steady-state inhibition) obtained when 1.7 μM MtAHAS is inhibited by 50 μM Q1 (Fig. 6B) and is −0.01443 AU/min, which represents 74% inhibition as the velocity before adding Q1 is −0.055 AU/min. This can be interpreted as 26% of the enzyme being reduced and 74% being oxidized. As the estimated $K_{m}$ of Q1 is 240 μM, it can be deduced that 17% of enzyme binds Q1 when its concentration is 50 μM. Therefore, at equilibrium, $(k_{ox} \times 0.17 \times 26) = (k_{act} \times 74)$. Because $k_{act}$ of MtAHAS is equal to 0.28 min⁻¹ (at the same experimental temperature (30 °C), Fig. 4), the value of $k_{ox}$ is estimated to be 4.7 min⁻¹ at 30 °C.

Molecular Docking of Q1 in ScAHAS (Fig. 10)

The ScAHAS-Q1 complex was obtained by molecular docking simulation of Q1 in the crystal structure of ScAHAS in complex with BSM (PDB code 5FEM) using the ChemPLP fitness of GOLD (15). The pose of Q1 in the binding pocket was selected according to the best score value function of ChemPLP and the binding free energy changes ($\Delta G$). The structure refinement and energy minimization of the ScAHAS-Q1 complex was calculated with the YASARA Energy Minimization Server (16).

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