Steady-State Fluorescence Properties of *S. solfataricus* Alcohol Dehydrogenase and its Selenomethionyl Derivative

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Alcohol dehydrogenase from the thermoacidophilic Sulfolobus solfataricus (SsADH) is a thermophilic NAD+-dependent homotetrameric zinc enzyme whose crystal structure has been recently determined at 1.85 Å resolution by using a selenomethionine-substituted enzyme. In this report the steady-state fluorescence properties of SsADH are related to the two fluorophores Trp95 and 117 located inside the monomer structure and whose indole ring centers are at 5.7 Å to each other. The relatively blue emission of the enzyme ($\lambda_{max} = 320$ nm) is due to the highly hydrophobic character of the microenvironment determined by six and seven non-polar residues in close contact (<7 Å) with Trp95 and 117, respectively. However, the contribution of the two residues to intrinsic fluorescence appears different since the Trp95 and 117 indole rings are found in the vicinity of five and one polar residue side chains, respectively. The fluorescence intensity of the selenomethionine-substituted enzyme is found to be 40% lower than that of the natural enzyme. Moreover, four out of the nine methionine residues per monomer are found in the vicinity (<6 Å) of as many tyrosine residue side chains, while no methionine-tryptophan interaction is present in the structure. Presumably, selenium acts as a quencher of the nearby tyrosine emission more efficiently than does sulfur, due to its larger electron cloud and polarizability. It cannot be excluded that an effect of selenium is to stabilize the tyrosinate ion allowing a more extended delocalization of the negative charge. Therefore, the decreased tryptophan emission of the seleno-protein would reflect the lower quantum yield of the tyrosine in its ionized state.

KEY WORDS: Archaeal alcohol dehydrogenase; NAD(H) quenching; sulfur/selenium quenching; sulfur-aromatic interactions.

INTRODUCTION

Alcohol dehydrogenase from the thermoacidophilic sulfur-dependent crenarchaeon *Sulfolobus solfataricus* (SsADH) is a thermophilic NAD+-dependent homotetrameric zinc enzyme endowed with remarkable thermostability and broad substrate specificity. So far, this is the only zinc-dependent alcohol dehydrogenase extracted from Archaea, a very peculiar kingdom of living organisms, which contains highly specialized organisms that can live both in unusual habitats (hot springs, sulfur springs, volcanic areas etc.) and in normal environments [1,2]. Cloning and overexpression in *E. coli* of the SsADH gene, as well as the kinetic and thermostability properties of the enzyme have been reviewed [3]. The 3D structure

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of the apo form, which lacks coenzyme, has been recently determined at 1.85 Å resolution by using a selenomethionine-substituted enzyme [4]; furthermore, the crystallization studies of the holo form are in progress. However, a homology-based 3D model of the apo and holo form of SsADH which is consistent with experimental data was computed using the 3D structures of horse liver, *Thermoanaerobacter brockii* and *Clostridium beijerinckii* ADH [5]. SsADH is a dimer of dimers which can be denoted AB and CD; each monomer comprising two domains separated by a cleft [4]. The subunit (347 residues, 37,588 Da) contains two tryptophan residues, Trp95 and 117, both located in the catalytic domain and thirteen tyrosine residues dispersed throughout the structure. The presence of an additional tyrosine residue in the mutant Asn249TyrSsADH has been described as increasing thermostability and catalytic activity other than the emission fluorescence of the enzyme [6].

In this paper the steady-state fluorescence properties of SsADH are correlated to the location of the two fluorophores and the significant quenching of protein fluorescence which occurs upon Se- for sulfur-methionine substitution is associated with the sulfur-aromatic interactions present in the enzyme structure.

MATERIALS AND METHODS

Chemicals

NAD$^+$ and NADH were purchased from Applichem (Darmstadt, FRG). L-tryptophan, L-tyrosine and L-isoleucine were from Aldrich (San Diego, CA). L-methionine and Se-L-methionine were from Sigma (St. Louis, MO). Other chemicals were A grade substances from Sigma or Applichem. Solutions of NADH were prepared daily in 20 mM Tris-HCl, pH 8.2, while solutions of NAD$^+$ were prepared in water, adjusted to approximately pH 6.5 with dilute NaOH and stored frozen at $-20°$C. The stability of the coenzyme solutions was checked by determining the absorption quotients as indicated by the manufacturer. All solutions were made up with MilliQ water.

Protein Purification

The native and selenomethionine recombinant wild-type SsADH were prepared according to the method described in [3] and [4], respectively. The mass spectra of the selenomethionyl protein confirmed 96% substitution Met→Se-Met [4]. Both homogeneous natural and Se-protein were stored at $-20°$C in 20 mM Tris-HCl, pH 8.5, in the absence and the presence of 10 mM 2-mercap-

toethanol, respectively. The protein concentration was determined as described in [3].

Fluorescence Studies

Steady-state emission and excitation spectra were recorded with a JASCO FP-777 spectrofluorometer, using a quartz cuvette containing 500 µl of solution and thermostated with a temperature regulated cell holder. The buffer used was 50 mM glycine-NaOH, pH 9.8 or 50 mM Tris-HCl, pH 8.8. The protein concentration ranged from 0.018 to 0.033 mg/ml (0.12 to 0.22 µM tetramer). Fluorescence quenching studies with free amino acids were performed by adding 12.5 mM methionine or Se-methionine to 8.4 µM tyrosine in 0.002 mM HCl, 50 mM Tris-HCl, pH 7-10, and 0.001 mM NaOH. Excitation and emission slit bandwidths were 5 and 10 nm, respectively, $\lambda_{ex.} = 280$ nm. Absorbances of all solutions at the exciting wavelength never exceeded 0.1, except for the Se-methionine-tyrosine mixtures whose fluorescence was corrected for the inner filter effect. Studies of the temperature dependence of SsADH fluorescence were performed allowing the samples to stand at the desired temperature for 5 min. Temperature was controlled in the cuvette and measured in all experiments to within $\pm0.2°$C. Fluorescence contributions from buffer components were subtracted from all the spectra. Each spectrum was obtained in duplicate or triplicate with virtually identical results.

Circular Dichroism Studies

CD data were collected at 25°C with a JASCO J-710 spectropolarimeter equipped with computer-controlled temperature cuvette holders kept under constant N$_2$ flux, according to the protocol described in [6]. Final spectra, representing the average of at least five tracings, were corrected for the buffer.

Structural Analysis

Analysis of characteristics of the surrounding environment of tryptophan and methionine residues was done on the basis of the atom co-ordinates of the apo SsADH [4] using the program Swiss-PdbViewer v3.7 [7]. The side-chain microenvironment of the tryptophan residues was determined taking into account all atoms that can contact the indole ring within a distance of 7 Å according to the criterion followed by [8]. Solvent exposure of residues was evaluated with the DSSP program [9]. This method yields the accessibility in Å^2 and establishes at 16 the percentage of accessibility with respect to the free

side-chain of residue to discriminate between an exposed (>16%) and a buried (<16%) residue.

RESULTS AND DISCUSSION

Figure 1 shows the fluorescence emission spectra of SsADH apo form obtained at the excitation wavelength of 280 and 295 nm. The difference spectrum between the two spectra, which has the λ_{max} value centered at 310 nm, indicates that the contribution of tyrosine residues to bulk fluorescence is quite modest, i. e. the SsADH fluorescence emission, upon excitation at 280 nm, is dominated by tryptophan fluorescence. Furthermore, the λ_{max} value centered at 320 nm (Fig. 1, spectrum A) indicates that both tryptophans are quite shielded from the solvent. Typically, ionization of tyrosine produces a weakly fluorescent tyrosinate [10,11] and the pK_a for the ionization of tyrosine residues in most proteins usually lies in the range 9–12 [12]; this feature was analyzed in SsADH [6]. The emission intensity of SsADH monitored at 303 and 320 nm decreases as the pH increases from about 8 to 11.5 due to the formation of tyrosinate, and yields similar pK_a values, 10.3 ± 0.1 and 10.4 ± 0.1, respectively [6]. This indicates that the matrix structure supports most of the non-radiative energy transfer from tyrosine to tryptophan residues.
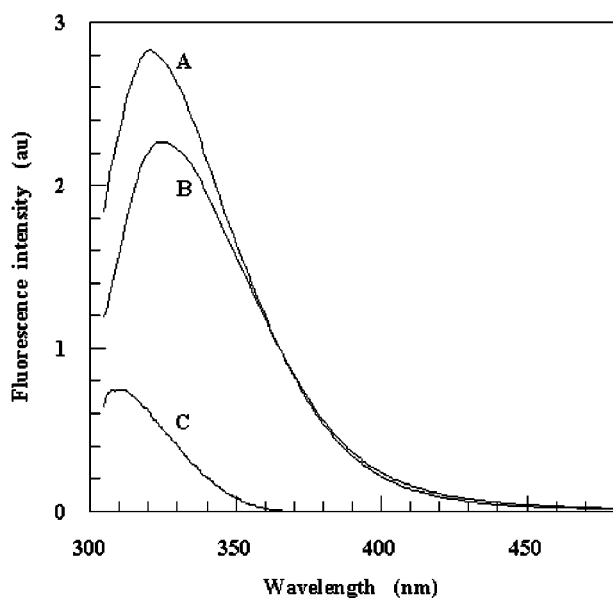

Fig. 2. Steady-state fluorescence emission spectra of SsADH in the absence (A and B) and presence (C and D) of saturating concentration of NADH. $_{ex}$ = 280 (A and C) and 295 nm (B and D). The enzyme concentration was 0.12 μM in 50 mM glycine-NAOH, pH 9.8. In the inset is the excitation spectrum of NADH bound to SsADH obtained with $_{em}$ = 435 nm. Excitation and emission slit bandwidths were 5 and 10 nm, respectively. Temperature, 25°C. The spectra are uncorrected.

Fig. 1. Steady-state fluorescence emission spectra of apo SsADH (0.16 M) excited at 280 (A) and 295 nm (B). Curve C is the difference spectrum between curve A and B. Excitation and emission slit bandwidth were 5 and 10 nm, respectively. Buffer used was 50 mM glycine-NaOH, pH 8.8. Temperature, 25°C. The spectra are normalized to unity at 365 nm.
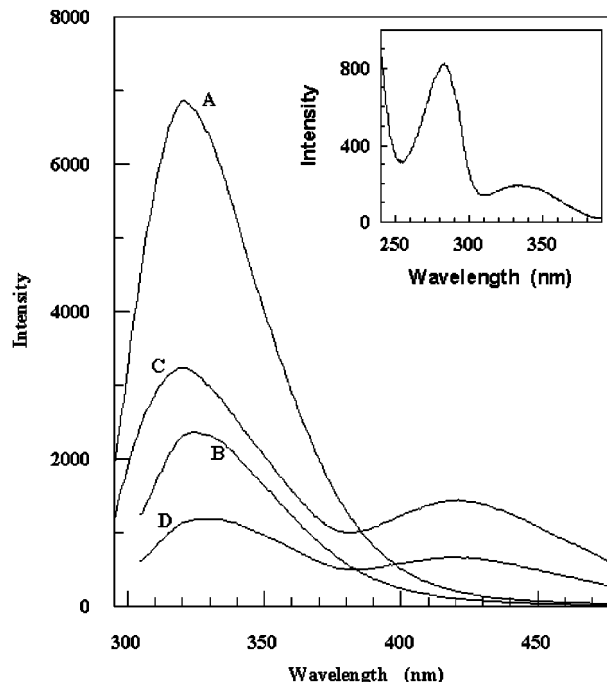
The addition of saturating concentrations of NADH to the SsADH apo-form results in 58% quenching of intrinsic fluorescence, with a concomitant appearance of a second peak at about 422 nm in the emission spectrum and a small, but discernible blue shift of λ_{max} of 1 nm (Fig. 2). This indicates that the interaction between the enzyme and coenzyme leads to slight shielding of tryptophan residue(s) and a radiationless transfer of the protein excitation energy, stimulating NADH emission at 422 nm. Accordingly, the spectral overlap of the emission spectrum of apo SsADH and the absorption spectrum of NADH are quite extended, from 290 to 420 nm (data not shown).

The excitation of the binary NADH-SsADH complex at 340 nm results in 9.1-fold enhancement of NADH emission and a blue shift from 458 to 429 nm of λ_{max} (data not shown). This large increase in quantum yield is due to three factors: unstacking of the adenine and nicotinamide rings of the coenzyme, shielding of the fluorophore from aqueous solvent, and tight binding, as resulted by docking the coenzyme molecule into the holo-form model of SsADH [5]. In the excitation spectrum of NADH bound to the enzyme (λ_{em} = 435 nm), a trypto-

phan band appears at 283 nm, suggesting that the quenching is caused mainly by energy transfer (Fig. 2, inset). Moreover, the position of λ_{max} at 320 nm of the difference spectrum between the apo and holo form spectra (not shown) suggests that the contribution of two tryptophans to energy transfer is similar.

NAD$^+$ binding to the apo-enzyme results in a 55–56% quenching of protein fluorescence, on excitation at 295 and 280 nm, presumably ascribable mainly to a conformational change, analogous to the case of horse liver [13] and yeast ADH [14]. This quenching is not accompanied by any shift, suggesting that there is no change in apolarity of the environment surrounding the tryptophan residue(s) (data not shown).

The lack of the 3D holo structure precludes any definite interpretation of difference in fluorescence data between the apo and holo form. Nevertheless, a comparison with the open and closed forms of the horse liver enzyme, performed to characterize the domain relative position in the SsADH apo structure, suggests that the conformational change occurring on the coenzyme binding to SsADH is characterized by a significant rotation of the catalytic domain relative to the coenzyme domain [4]. This movement should account for the quenching of tryptophan emission by approaching amide or carboxyl groups to the indole ring and/or rendering more favorable the fluorophore-quencher mutual orientation. It cannot be excluded that tyrosine ionization can occur upon the structural rearrangement thus determining part of the observed fluorescence quenching.

Inspection of the monomer 3D SsADH structure shows that Trp95 is surrounded by six hydrophobic residues and by five polar residues whose side chains are all within 7 Å of the heteroatoms on the indole ring (Table I). Trp117 is surrounded by seven hydrophobic residues and one polar residue (Table I). Trp95, Phe49, Ile120, Leu295, and Trp117 comprise the substrate-binding site, which is essentially hydrophobic as typical for ADHs [4]. The center of the Trp117 ring lies at a distance of 5.9 and 5.7 Å from the Phe49 and Trp95 ring center, respectively. In particular the two tryptophan residues belong to two short stretches of β-pairs connecting the terminal parts of the zinc structural lobe (residues 95–117) and they interact with each other by the preferred T-shaped orthogonal packing geometry (inter-planar angle = 75°). The Phe49-Trp117 pair shows a similar arrangement, with an inter-planar angle of about 60° (Fig. 3).

It is evident that Trp95 and 117 residues are included in an aromatic cluster that determines the high and blue-shifted emission of the protein. However, a thorough analysis of the microenvironment of the two tryptophan

Table I. Distance between the Tryptophan Indole Ring and the Amino Acid Side Chain involved in the Microenvironment of Tryptophan Residue in SsADH.

Residue	Trp95 R (Å)[a]	Residue	Trp117 R (Å)
Phe-49	(6.4–8.6)	Phe-49	(5.6–6.6)
Pro-94	(3.5–5.1)	Leu-58	(6.1–7.8)
Ile-120	(6.3–8.2)	Pro-115	(3.8–6.2)
Ile-157	(5.4–7.0)	Ile-120	(5.5–6.9)
Val-296	(3.4–4.0)	Leu-295	(6.2–7.5)
Trp-117	(5.5–7.0)	Val-296	(6.0–7.0)
His-68	(5.6–7.5)	Trp-95	(4.0–7.4)
Asn-93	(5.1–7.2)	Asn-121	(5.4–6.3)
Thr-153	(6.8–8.2)		
Cys-154	(5.8–5.9)		
Thr-158	(6.4–8.7)		

[a]R is the distance between the side chain of the indicated residue and the geometric center of the tryptophan indole ring (minimal and maximal values are given). There no residues of adjacent subunits in the microenvironment of either tryptophan residue.

residues suggests that they could contribute to bulk fluorescence to varying extents.

The tetramer structure shows that the values of relative accessibility for the Trp95 and 117 side chains are 1 and 7%, respectively. So Trp95 is completely buried but lies in a relatively polar environment due to the presence of the polar groups of four residues in its vicinity. One of these—Cys154—has the sulfur atom, an effective quencher [11], at close distance from the Trp95 indole ring, which in turn is in close contact to the amide group of Asn93, the atom OG1 of Thr158 and Thr153 of (Table I and Fig. 3). The His68 ring, whose nitrogen NE2 is bound to the catalytic zinc atom, could affect the Trp95 emission to some extent, depending on its orientation and ionization state [15]. However, Trp95 lies between the Phe49 aromatic ring and Pro94 whose ring is roughly parallel to that of the indole, suggesting that oscillations of Trp95 are restricted (Fig. 3).

On the other hand, Trp117 is less shielded than Trp95 but interacts with only the Asn121 amide group. Furthermore, Trp117 is very close to Pro115 but the two rings planes are roughly perpendicular to each other with the indole nitrogen pointing towards the CB atom of the proline ring (Fig. 3).

Finally, Trp95 lies in a relatively rigid environment but suffers relatively large quenching effects; on the other hand, Trp117 is slightly exposed to solvent and experiences the quenching effect of a near polar group only. The rings of the two tryptophans appear practically perpendicular to each other (Fig. 3). This implies that a
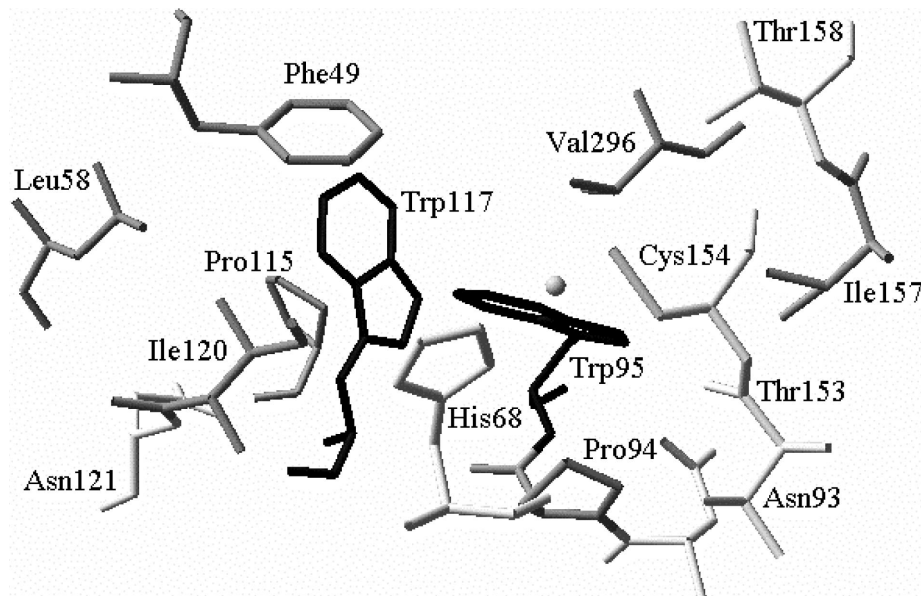
Fig. 3. Peculiarities of the microenvironment of the tryptophan residues of apo *Sulfolobus solfataricus* ADH. Trp95 and 117 are reported in black, the non-polar and polar residues are reported in dark and light grey, respectively. The grey sphere represents the catalytic zinc atom. The figure was drawn by Swiss-PdbViewer v3.7 (7).

not very high non-radiative energy transfer should occur between them, due to an unfavourable mutual orientation factor.

The model of SsADH is consistent with the burial of both tryptophan residues in the apo structure as well as with the negligible blue shift of the λ_{max} emission occurring on coenzyme binding. In fact, the relative accessibility values of the Trp95 and 117 side chain for the apo-form are 9 and 20%, respectively, in fairly good agreement with the data from the crystal structure, and 5 and 21%, respectively, for the holo-form of the model [5]. Moreover, docking of the coenzyme molecule to the model of holo SsADH shows that the indole rings of Trp95 and 117 are approximately 6 and 16 Å distant from the nicotinamide moiety of the coenzyme, respectively [5]. Therefore, on the basis of the accuracy of the model, both tryptophan residues contribute toward the energy transfer to the reduced coenzyme with different levels of efficiency. However, a quantitative estimate of the separate contributions to energy transfer would require the knowledge of the relative angles of the transition dipole moments of NADH and tryptophan that will be achievable only once the holo enzyme structure is available.

Within the monomer the two tryptophan residues are quite close to each other, 5.7 Å. On the other hand, the distances between Trp95 and 117 of monomer A and their homologues in monomers B, C and D range from 30 to 50 Å. This indicates that the energy transfer process

between tryptophan and NADH should occur more efficiently within the subunit than among the subunits.

Thermophilicity of SsADH is remarkable; in fact, the enzyme activity increases with temperature up to an instrumental limit of about 95°C, and the half-life determined at 70°C is 5 hours [6]. The Arrhenius plot is linear from 20°C to the optimum temperature for the forward and backward reaction [6], suggesting that the maximum steady-state in each direction is controlled by one rate constant and increases with temperature, as with ordinary chemical reactions; moreover linearity seems to exclude any temperature-dependent conformational change of the overall structure. The effect of temperature on the SsADH fluorescence has been investigated up to an instrumental limit of 70°C (Fig. 4). Fluorescence intensity decreases by about 50% on excitation at 280 and 295 nm as temperature rises from 10 to 70°C, whereas the position of the emission maximum shifts by 2 ± 0.5 nm toward longer wavelength in both cases (Fig. 4, inset). The slight red shift reflects the rigidity and hydrophobic character of the aromatic cluster where Trp95 and 117 are located. Evidently, gradual thermal expansion of the molecule over the temperature range could permit only a very small access of solvent molecule to tryptophan residue. The higher decrease in emission observed exciting the protein at 280 nm could be the result of an increase in the freedom of motion of some tyrosine residues that at low temperature are held in an orientation particularly
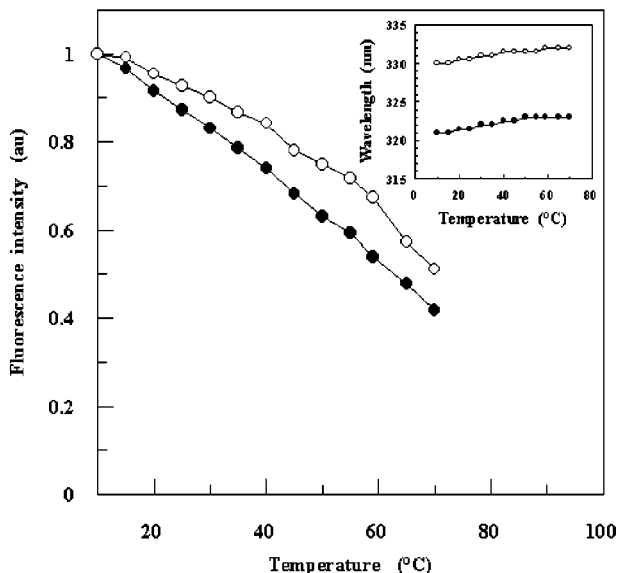
Fig. 4. Effect of temperature on steady-state fluorescence emission of apo SsADH excited at 280 (black symbols) and 295 nm (white symbols). In the inset is shown the temperature dependence of the emission fluorescence spectral position of the enzyme excited at 280 (black symbols) and 295 nm (white symbols), respectively. Protein concentration and buffer as for Fig. 1.

favorable for a dipole-dipole energy transfer to tryptophan. It is important to mention that the dichroic activity at 222 nm of SsADH does not change at all in the temperature range 20–80 °C, consistent with the marked structural stability of the enzyme [3].

Treating the enzyme with 4 M guanidinium chloride at 30°C results in complete quenching of emission at 319 nm and the appearance of a band centered at 345 nm as well as 90% loss of the catalytic activity [6]. However, 0.25 M denaturant yields a 110% enhancement in both activity and emission, suggesting that a slight weakening of some native structure interactions quenching the tryptophan occurs on structural loosening by moderate denaturant concentrations. A large enhancement of fluorescence of chemically unfolded homeodomains has been related to the presence of an indole NH··· aromatic π hydrogen bond which is responsible for the quenching of tryptophan emission in the native protein [16]. Inspection of the apo SsADH structure shows that the orientation of the indole rings of Trp95 and 117 with respect to the nearby aromatic rings (Fig. 3) is not favorable for *this* type of interaction. On the other hand, whether or not the conformational change accompanying the coenzyme binding to the apo enzyme can result in this peculiar interaction remains an interesting hypothesis to verify on the holo-enzyme 3D structure.

The SsADH tetramer contains 36 methionine residues that were substituted successfully for Se-Met using a methionine auxotropic expression system, in order to assist in solving the crystallographic 3D structure [4]. On occasion, the availability of the selenomethionyl derivative of the protein prompted us to compare its spectroscopic properties with those of the natural protein. The overall secondary structure of the modified enzyme was not found to be significantly affected by the presence of Se-Met residues as indicated by the far-UV CD spectra that is superimposable to that of the natural enzyme in the 196–250 nm region (data not shown). However, we cannot exclude that local conformation changes may, of course, occur while leaving the secondary structure blocks essentially unaltered.

The fluorescence emission of the Se-protein is 3 nm red shifted and decreased by 41 and 31% with respect to the natural protein, on excitation at 280 and 295 nm, respectively, (Fig. 5). The spectral shift suggests a slight increase in polarity of the tryptophan(s) microenvironment; the decrease in emission, instead, raised the question whether it could be ascribable to a quenching effect of Se atoms on the fluorophore groups of the SsADH molecule or to subtle changes in backbone conformation affecting the peptide bond quenching, or to both these effects.

Therefore, inspection of the apo SsADH 3D structure was carried out to find intra- and interchain sulfur-aromatic interactions between methionine and tryptophan
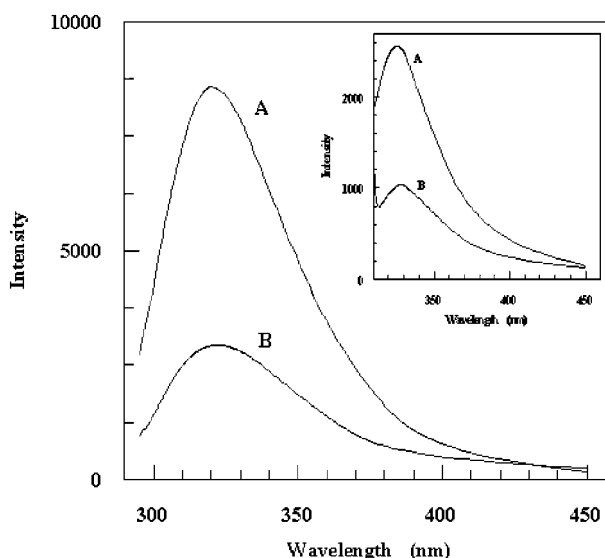

Fig. 5. Steady-state fluorescence emission spectra of apo SsADH (A) and apo SeMet-SsADH (B) upon excitation at 280 (main panel) and 295 nm (inset). Buffer, 50 mM Tris-HCl, pH 8.8; others experimental conditions as for Fig. 1. The spectra are uncorrected.

and tyrosine. Computer analysis searched for sulfur atoms within a distance of less than 6 Å, a range that includes all sulfur atoms in van der Waals' contact with the aromatic ring, as specified in [17]. Data analysis, which also includes interactions involving phenylalanine and histidine, are given in Table II. Four out of nine methionine residues present per subunit interact through the sulfur atom with the aromatic rings of tyrosine residues, and these interactions are found in the interior of the protein as a consequence of the non-polar character of aromatic and sulfur-containing amino acids. Remarkably, no methionine-tryptophan interaction within the 6 Å range is present in the SsADH structure. Met44 is the nearest to the tryptophans, since the sulfur atom is 11 and 9 Å from the Trp95 and 117 indole center, respectively. Also important is the minor quenching of emission obtained exciting the protein at 295 with respect to 280 nm. This suggests that the decrease in the SeMet-SsADH emission is due to a quenching effect that the Se atom exerts mainly on the nearby tyrosine residues, since tryptophans are quite apart. The quenching mechanism should be dynamic (collisional) or static in nature, since both require molecular contacts between the quencher and the fluorophore [11]. The relatively larger electron cloud carried by Se and its larger polarizability with respect to the sulfur atom would explain the higher quenching efficiency of Se. It is not excluded that one of the effects of Se is to stabilize the tyrosinate ion allowing a more extended delocalization of the negative charge. Consequently the decreased emission of tryptophan in SeMet-SsADH could reflect the lower quantum yield of the tyrosine in its ionized state.

Of interest is the experimental result reported in Fig. 6. Se-methionine affects the tyrosine emission more than S-methionine as pH increases; however, an inversion from quenching to enhancement of emission seems to occur at pH 9 and 8, respectively. This effect could due to the prevailing of the neutral on the charged form of
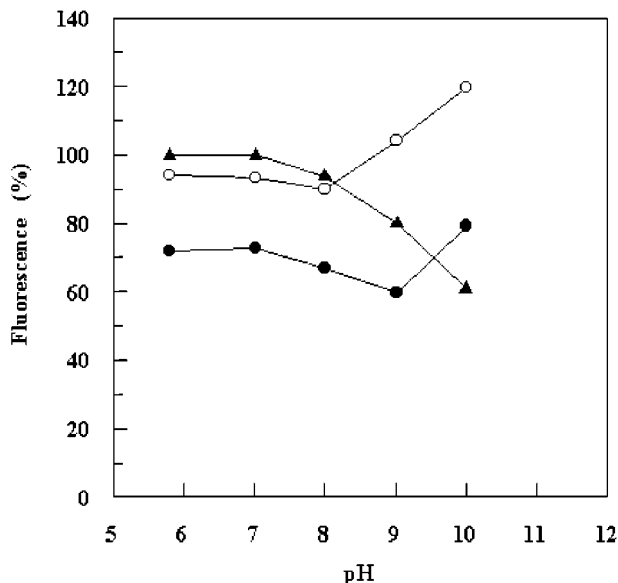

Fig. 6. Quenching effect of S-methionine (white circles) and Se-methionine (black circles) on the tyrosine fluorescence at different pH values. The tyrosine emission measured at 303 nm in the presence of the quencher is presented as percent relative to the value in the absence of the quencher. The dependence of tyrosine emission on pH (triangles) is also shown as percent relative to the value measured at pH 5.8. Temperature, 25°C. For details see Material and Methods.

methionine amino group and this should limit the excited-state ionization of tyrosine through the hydrogen bonds formation with the phenolic hydroxyl group.

In binding studies of tryptophan free-calmodulin to target proteins Se-methionine has been found to be a more efficient fluorescence quencher than S-methionine [18]. In this case the quenching effect only influences the single Trp residue of the bound peptide which is at less than 7 Å from the sulfur atoms of four methionine residues of the calmodulin molecule.

In addition to methionine-aromatic interactions, there are also cysteine-aromatic interactions carried out by three out of five cysteine residues present per monomer. The sulfur atoms of Cys38 and 154, both ligands of the catalytic zinc, and Cys101, a ligand of the structural zinc, are in close contact to the aromatic ring of His68, Trp95 and Tyr103, respectively. Sulfur-aromatic interactions have been observed in the hydrophobic core of proteins and have been proposed to contribute to protein structure stability [17, 19]. More recently, evidence for a strong sulfur-aromatic interaction derived from crystallographic data has been presented [20] which indicates that a single sulfur-aromatic interaction can stabilize a complex by 1–2 kcal/mole. Present analysis is limited only to the sulfur atom-aromatic ring distance and does not concern details about the preferred geometry of con-

Table II. S-Met–Aromatic Interactions in SsADH.[a]

	Tyr	Phe	His
Met 1	129 (5.3)		
Met 44		49 (7.0)	68 (5.6)
Met 138	84 (5.4)		
Met 269	267 (5.8)	291 (5.7)	
Met 306	139 (5.8)	302 (4.9)	

[a]The distance in Å is reported in parenthesis and is expressed as the average distance from the methionine sulfur atom to the atoms on the aromatic ring. The values were measured by Swiss-PdbViewer v3.7 (7). No sulfur-aromatic interaction was found between subunits.

tact [20], however it is plausible that these special interactions contribute substantially to stabilize the folded conformation of the archaeal enzyme.

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