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DOMAINS IN BOVINE SERUM AMINE OXIDASE

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Analysis of the thermal unfolding of bovine serum amine oxidase by differential scanning calorimetry reveals for the dimeric protein a four domain structure consisting of two sets of domains. Each set contains two domains of similar size. The two smaller domains, in contrast with the larger ones, greatly differ in thermostability. Removal of copper changes the calorimetric pattern dramatically. The findings confirm that the metal cofactor plays a structural role. Since the enzyme contains two copper atoms and only one titratable carbonyl group, the calorimetric pattern suggests that the difference in thermostability of the two small domains might be due to the presence of a single organic cofactor.

Copper amine oxidases (AO) are enzymes which catalyze the oxidative deamination of primary amino groups of biogenic amines. These enzymes are composed of two subunits containing only one titratable organic cofactor, pyrrolquinolinequinone (PQQ) or a closely similar derivative (1,2).

The enzyme molecules also contain two copper atoms both necessary for activity. At least one copper seems to be in the neighborhood of PQQ, but not very close to the carbonyl group (3,4). Recently the possible involvment of PQQ ring nitrogens in the coordination of copper seems to have been ruled out (5).

The copper sites are not identical (6,7,8). Cu(II) may play a role in the oxidation of the reduced form of these enzymes (7,8,9,10,11,12), and appears to be involved in the reaction with oxygen. A role of copper in mantaining the correct conformation of the enzymes has been suggested (4,8,10,13).

In order to gain further information on the relationship among PQQ, copper and protein, the thermal unfolding of bovine serum

Abbreviations used : BSAO, bovine serum amine oxidase; PQQ, pyrrolquinolinequinone; DSC, differential scanning calorimetry.

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amine oxidase (BSAO) has been investigated by differential scanning calorimetry (DSC) of the native and copper free enzyme. DSC has been used as a tool for obtaining information about structure in several proteins (14,15,16,17). Resolution of complex thermograms into Gaussian functions (deconvolution) has lead to the definition of structural domains in a number of cases, even when little structural information was available(18).

Materials and Methods

All reagents were of analytical grade.
BSAO was prepared as a homogeneous protein as previously described (19). Protein concentration was determined spectrophotometrically using E mg/ml = 1.74 at 280 nm(10).
Cu II content was determined by the biquinolyl method (20), by e.p.r. spectra, and by atomic absorption spectrophotometry. A value of 2.0 g.atoms of Cu (II) per mol of protein was always obtained.
PDQ content was checked measuring the band at 445 nm formed by reaction of the protein with stoichiometric phenylhydrazine (21). One mol of adduct per mol of protein was always found.
Enzyme activity was assayed spectrophotometrically according to Tabor et al.(22). Samples used had always a specific activity of .24 umol/min mg or more.
Copper depleted enzyme was prepared according to Suzuki et al.(10). The preparations contained 2% residual copper.
Calorimetric measurements were performed using a high resolution MC-2 scanning calorimeter (Microcal, Amherst, MA) equipped with the DA-2 digital data acquisition system, at a heating rate of 60° C/h.
Enzyme solutions of about 2 mg/ml in 0.1 M phosphate buffer, pH 7.2, were routinely employed, except when otherwise stated.
Thermograms were always corrected by subtracting the instrumental base line obtained by filling both cells with the buffer used.
Integration and deconvolution procedures were carried out using software provided by Microcal Co. after normalizing the data for protein concentration and subtracting as chemical baseline the straight line connecting the initial and final temperatures of the overall transition(23).

Results

A typical pattern for the thermally induced unfolding of native BSAO is shown in Fig.1.
No precipitation was observed after heating the sample to 97°C. No endotherm was noticed upon reheating the sample.
Values of Tm (temperature at which heat capacity exhibits a local maximum), ΔH_c (calorimetric denaturation enthalpy change), ΔH_{vH} (van't Hoff denaturation enthalpy change) and ΔC_p (heat capacity

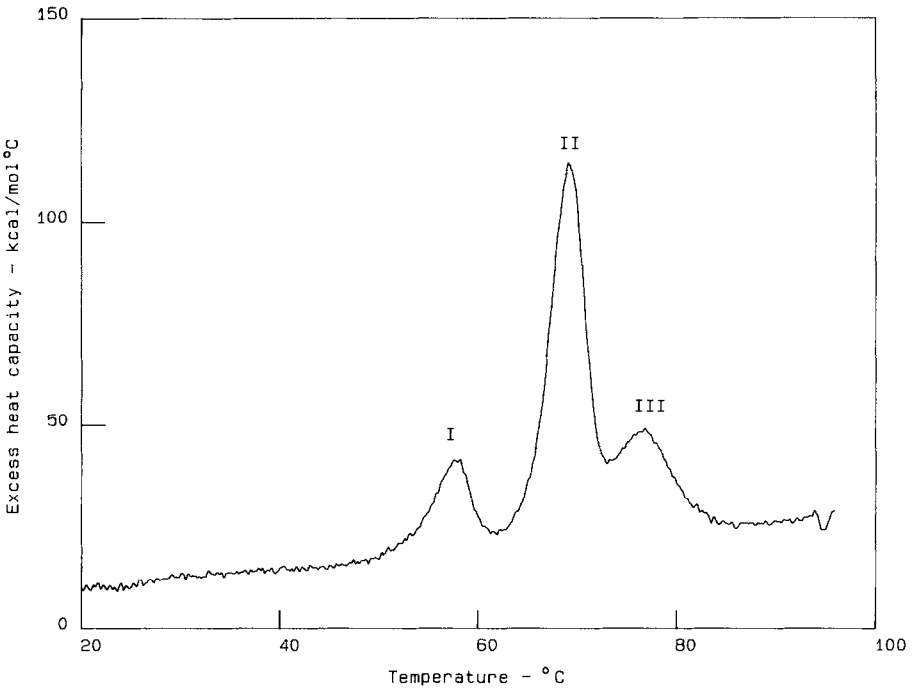

Fig.1. Temperature dependence of molar partial heat capacity of BSA0.

change upon denaturation estimated for the overall process) are given in Table 1.

The temperature dependence of partial heat capacity does not vary with protein concentration, since thermograms obtained on the same sample before and after tenfold dilution are related purely by a tenfold scaling factor. The aggregation state of the protein should not therefore change during denaturation.

The calorimetric behavior of native BSA0 is not appreciably altered by reaction of its cofactors with small ligands (e.g. phenylhydrazine for the prosthetic group and diethyldithiocarbamate for copper).

TABLE I

Calorimetric values for BSA0 denaturation

	Tm °C	ΔHc kcal/mol	ΔHvH kcal/mol	ΔCp kcal/mol°C
overall		879±9%		8.2±10%
peak I	57.4±0.1	149±11%	122±9%	
peak II	69.3±0.3	542±15%	206±4%	
peak III	77.8±0.5	165±7%	127±8%	

Values are the average of three independent determinations. Error is expressed as average deviation for Tm and as percent average deviation for ΔH and ΔCp.

A denaturation curve with three distinct peaks, as the one shown in Fig.1, usually indicates the presence of different structural domains in the protein. The ratio of ΔH_{vH} to ΔH_c calculated on the basis of the subunit molecular weight is much higher than unity (e.g.1.6) for peaks I and III: this means that the molecular weight of the cooperative unit is necessarily greater than that of a single monomer (24). The values in Table I were consequently calculated on the basis of the molecular weight of the dimer. The data show that in this case the ratio of ΔH_{vH} to ΔH_c, which should approximate unity for a two-state transition (25), is .8 for peaks I and III and .4 for peak II. This suggests that peaks I and III, within the limits of experimental uncertainty, represent two-state transitions of structural domains. Peak II, instead, represents a more complex process, presumably the denaturation of more than one folding domain in a single cooperative unit.

The thermograms were analyzed by different deconvolution procedures: the best fit which accounts for their shape requires a minimum of four two-state independent transitions. The thermodynamic values derived for the component transitions are given in Table II, and the shape of the four individual curves and of the resulting one are shown in Fig.2.

When the copper-free derivative of BSAO was heated in the differential scanning calorimeter only one broad peak was observed (Fig.3) which is not amenable to deconvolution with less than eight two-state component transitions. Table III gives the calorimetric values relative to copper-depleted BSAO: the data must be taken with caution, since they are greatly influenced by the choice of the chemical baseline.

TABLE II

Thermodynamic parameters for fitting the calorimetric data of native BSAO to the sum of four independent two-state transitions

peak	Tm °C	ΔH^a kcal/mol	%[b]
I	57.6	139	17
IIa	68.1	233	29
IIb	69.8	280	35
III	76.8	153	19

a) $\Delta H_c = \Delta H_{vH}$
b) percent of total ΔH.

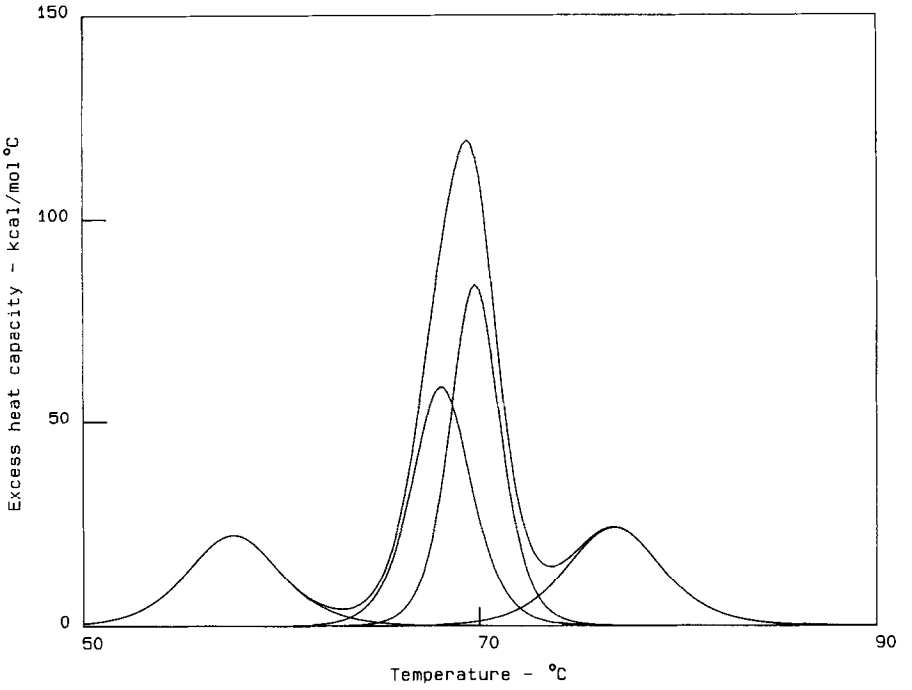

Fig.2. Deconvolution of BSA0 excess heat capacity function.

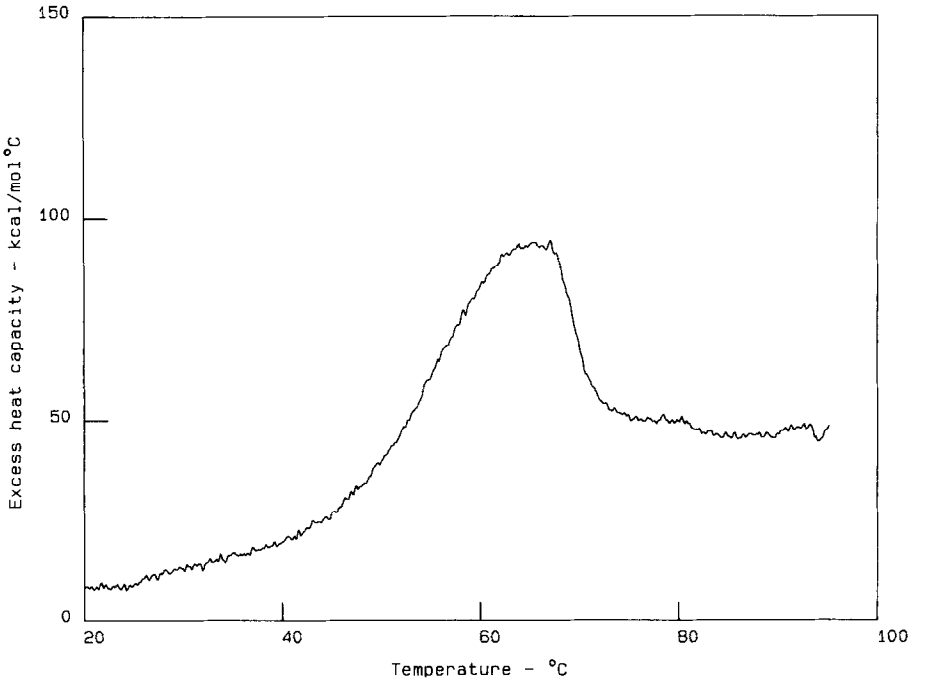

Fig.3. Temperature dependence of molar partial heat capacity of copper-depleted BSA0.

TABLE III

Calorimetric values for copper-depleted BSAO denaturation

Tm °C	ΔHc	ΔHvH	ΔCp
	Kcal/mol		Kcal/mol°C
63.4±0.2	1054±4%	41±7%	54±12%

Values are the average of three independent determinations. Error is expressed as average deviation for Tm and as percent average devation for ΔH and ΔCp.

Discussion

The thermal denaturation pattern of native BSAO shows three separated endotherms, whose calorimetric enthalpies are roughly in proportion 1:4:1. The total denaturation enthalpy and heat capacity of BSAO are around the average that has been found for compact globular proteins(25).

The complex thermal unfolding behavior of native BSAO gives some clues on the structural organization of the protein. The thermodynamic data reveal that the dimer is the fundamental cooperative unit. At least four different domains may be recognized: taking the transitions enthalpy change as an indication of domain size (26), we can define two sets of domains similar in pairs. The two large domains differ in thermostability by only 1-2 degrees, while the melting temperatures of the two smaller domains differ by twenty degrees.

Removal of copper displaces at least the two transitions with higher stability, and produces a broad peak, characterized by a greater increase in heat capacity than normally found for globular proteins (25).

The dramatic change in the endotherm after the removal of copper confirms an important structural role for the metal cofactor.

BSAO is a dimer containing two copper atoms and only one titratable organic cofactor. It is tempting to speculate that each monomer, in the presence of copper, folds in two different domains, comprising about 40 and 60% of the polypeptide respectively. The great difference in heat-stability characterizing the two small domains might then be tentatively ascribed to the presence of a single covalently bound organic cofactor.

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