CALORIMETRIC STUDIES ON THE BINDING OF IRON AND ALUMINIUM TO THE AMINO- AND CARBOXYL-TERMINAL FRAGMENTS OF HEN OVOTRANSFERRIN

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1. Introduction

The binding of iron(III) and other metal ions to ovotransferrin confers thermal stability on the protein [1]. This observation prompted the use of differential scanning calorimetry (DSC) for a more detailed study of the binding of Cu(II), Al(III), Fe(III), Zn(II) and Ga(III) to ovotransferrin [2–4]. The disappearance and appearance of the various liganded forms of a protein titrated with metal ions can be followed by DSC, provided that each species has a characteristic denaturation temperature.

Saturation of iron-free ovotransferrin with Fe(III) at pH 7.5 raises its denaturation temperature from 63–84°C. On partial saturation of the protein, intermediate endotherms (peaks of heat absorption) at 68°C and 77°C are observed, tentatively assigned to two monoferric species [3]. When Al(III) is added to the apoprotein to 50% saturation, the denaturation temperature is raised to 68°C. Further addition of Al has no effect on the thermal stability.

Here, we have examined the effects of Fe(III) and Al(III) on the thermal stability of the amino- and carboxyl-terminal fragments of ovotransferrin which can be isolated by proteolytic digestion [5,6]. Our results show that there is preferential binding to Fe(III) to the amino-terminal fragment, and that this fragment is more stabilized by Al(III).

2. Materials and methods

The amino(N)-terminal fragment of hen ovotransferrin was prepared by tryptic digestion of protein which had been 30% saturated with FeNTA [5]. The carboxyl (C)-terminal fragment was isolated by tryptic digestion of fully-saturated protein which had been depleted of its iron to 30% by treatment with citrate at pH 5 [6].

Iron(III) and aluminium(III) were added in the form of their nitrilotriacetate (NTA) complexes.

DSC thermograms (plots of heat flow as a function of temperature) were recorded on a Du Pont model 990 thermal analyser equipped with a DSC cell as previously described [3]. Calculated enthalpies of denaturation are probably good to only ± 20% in some cases. Sample sizes ranged from 17–24 μl. Reference material was an approximately equal weight of the appropriate buffer. The heating rate was 10°C/min for all experiments.

3. Results and discussion

The iron-free N- and C-terminal fragments are denatured at 56.5°C and 60.0°C, respectively (fig.1). When Fe(III) is added, the denaturation temperatures are raised to 77.5°C and 86.5°C. Thus, the fragments have different denaturation temperatures both with and without iron. But in ovotransferrin, both domains must denature simultaneously, as single endotherms are observed at 63°C and 84°C for the iron-free and iron-saturated forms, respectively [3]. (The two domains in iron-free human serum transferrin are denatured separately, since two endotherms are observed at 63°C and 73°C [7].) Enthalpies of denaturation and denaturation temperatures are listed in

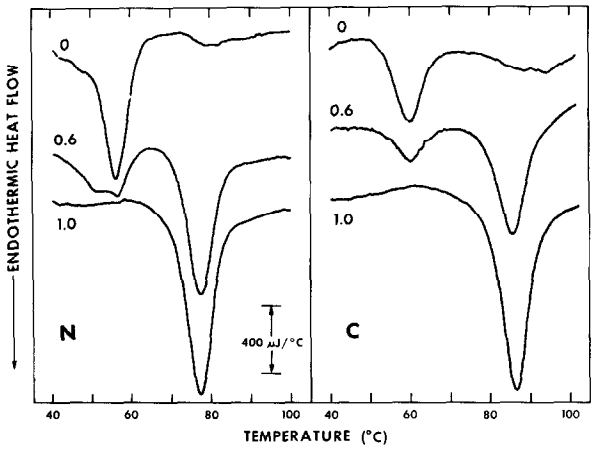

Fig.1. Iron-binding to ovotransferrin fragments. DSC thermograms for binding of FeNTA to N- and C-terminal fragments of ovotransferrin at pH 7.5 in 0.5 M Tris–Cl. Curves are marked with molar ratio of iron to fragment. Protein concentrations: (N) 23 mg/ml; (C) 19 mg/ml.

table 1. Note that the sum of the enthalpies of denaturation of the two fragments never exceeds half that of ovotransferrin, and is often considerably less. This suggests that a considerable amount of intramolecular interaction is eliminated by separating the two domains of ovotransferrin.

Table 1
Denaturation temperature and enthalpies of denaturation

Species (0.5 M Tris–Cl, pH 7.5)		T_d (°C)	ΔH_d[a] (10^6 J/mol)
apo-N, apo-C		57, 60	0.27
apo-ovotransferrin		63	1.33
Fe-N, Fe-C		77, 86	0.60
Fe_2-ovotransferrin		84	2.62
Fe-N, Fe-C	1 M	68, 78	0.25
Fe_2-ovotransferrin	$NaClO_4$	75, 79	1.3
Fe-N, Fe-C	2 M	63, 76	0.06
Fe_2-ovotransferrin	$NaClO_4$	68, 75	1.3
Al-N, Al-C		63, 62	0.29
Al_2-ovotransferrin		68	1.6

[a]Enthalpies of denaturation are given per mole of fragment (35×10^3 g) and per mole of ovotransferrin (77×10^3 g). The enthalpies of denaturation of the N- and C-fragments are similar, and are averaged in this table. Enthalpies of denaturation of ovotransferrin, except in perchlorate, are taken from references [2,3]

As the iron-saturated ovotransferrin fragments have substantially different denaturation temperatures, their relative affinity for Fe(III) can be studied by DSC. An equimolar mixture of the iron-free fragments gives a somewhat broad endotherm in the region of 58°C (fig.2), since the two apo fragments have similar denaturation temperatures. However, addition of FeNTA to about half-saturation of the binding sites produces an endotherm at 77°C, characteristic of the iron-saturated N-terminal fragment. Addition of FeNTA to 100% saturation results in the disappearance of the low temperature endotherm and the apperance of an endotherm at 86°C, characteristic of the iron-saturated C-terminal fragment.

There is much conflicting data on the relative affinities of the two binding sites of ovotransferrin for iron, and for their order of occupancy [8]. Until now

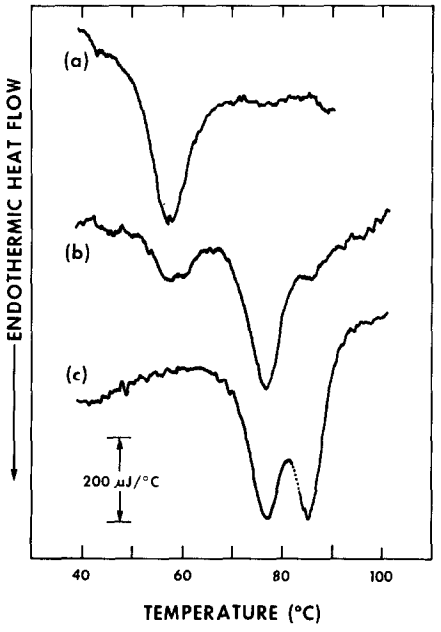

Fig.2. Competitive iron-binding experiment. DSC thermograms for binding of FeNTA to a mixture of N- and C-terminal fragments of ovotransferrin at pH 7.5 in 0.5 M Tris–Cl. (a) No FeNTA added; (b) approximately half-saturated with FeNTA and (c) full saturation with excess of FeNTA. Protein concentrations: (a,b): (N) 8.4 mg/ml; (C) 7.0 mg/ml. (c): (N) 10.0 mg/ml; (C) 9.6 mg/ml. An extraneous electrical transient has been removed from curve (c) – dotted region.

it has been difficult to distinguish between the case of intrinsically different sites or a difference induced by interaction between sites (cooperativity) or between the protein and chelating agent. Our results on the isolated fragments show that at pH 7.5 the N-terminal site has an intrinsically higher affinity for Fe(III). This conclusion is based on the assumption that the fragments we prepare are substantially the same as the iron-binding domains of ovotransferrin [9].

The chaotropic agent perchlorate is known to affect the epr spectrum of fully-saturated ovotransferrin [10], probably by altering the conformation of the protein. An epr study on the isolated fragments [11] has shown that they have slightly different spectra but in the presence of 1.4 M perchlorate the difference is abolished. Perchlorate decreases the thermal stability of ovotransferrin. At a concentration of 2 M, the endotherm for fully-saturated ovotransferrin is resolved into two peaks at 68.0°C and 75.0°C. This suggests that in 2 M perchlorate the two domains denature independently or at least that the interactions between them have been substantially diminished [12]. We have examined the effect of perchlorate on the thermal stability of the N- and C-terminal fragments and their relative affinity for Fe(III). In the presence of 1 M perchlorate, both iron-free fragments have broad, poorly defined endotherms in the region of 40–50°C. A decrease in the denaturation temperatures and enthalpies of denaturation of the iron-saturated fragments is also observed under these conditions. However, sharp endotherms are observed at 68°C for the iron-saturated N-terminal fragment, and at 78°C for the iron-saturated C-terminal fragment (fig.3). In the presence of perchlorate, the higher affinity of the N-terminal fragment for Fe(III) still remains, since when iron is added to a mixture of the iron-free fragments, only the endotherm at 68°C appears at low iron saturation (fig.3). At 2 M perchlorate, denaturation temperatures and enthalpies of denaturation of the fragments are further decreased (fig.4). In this solvent, the enthalpies of denaturation of the fragments are considerably smaller than those of the domain of ovotransferrin (table 1). Thus, some interactions between domains still seem to be present in ovotransferrin in 2 M $NaClO_4$, even though the domains are denatured at different temperatures.

Ovotransferrin is stabilized by addition of Al(III), since its denaturation temperature is raised from

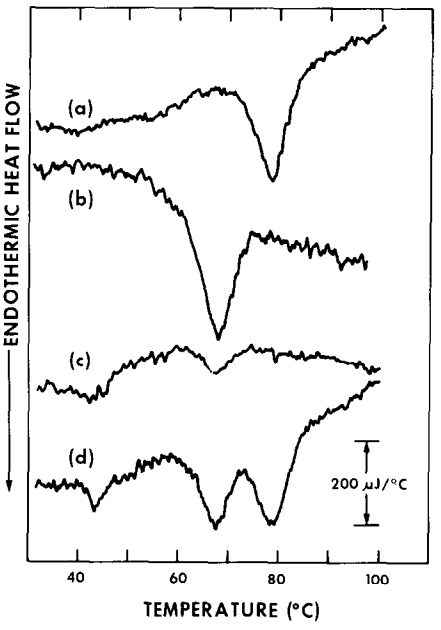

Fig.3. Iron-binding in 1 M perchlorate. DSC thermograms of the separate N- and C-terminal fragments of ovotransferrin and an equimolar mixture of fragments at pH 7.5 in 0.5 M Tris–Cl, 1 M $NaClO_4$, 0.02 M CO_2. Protein concentrations: (a) Iron-saturated (C) 9.6 mg/ml; (b) iron-saturated (N) 9.5 mg/ml; (c,d) mixture of N- and C-fragments, each at 7.7 mg/ml; (c) 0.19 FeNTA/site; (d) 1.2 FeNTA/site. An extraneous electrical transient has been removed from curve (c) – dotted region.

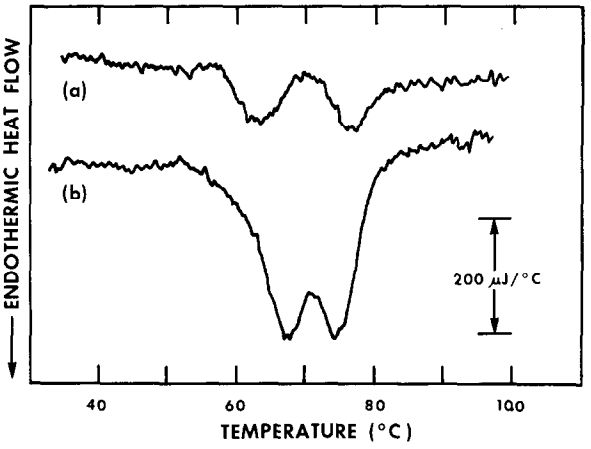

Fig.4. Comparison of iron-saturated N- and C-fragment mixture with iron-saturated ovotransferrin in 2 M $NaClO_4$. DSC thermograms in 0.5 M Tris–Cl, 2 M $NaClO_4$, 0.02 M CO_2, at pH 7.5. (a) N- and C-fragment mixture, each at 15.3 mg/ml; (b) ovotransferrin, 12.4 mg/ml. Curve (a) has been redrawn to correct for a very distorted baseline.

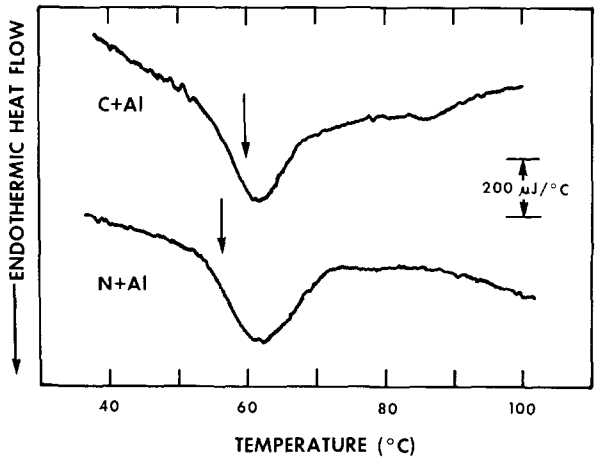

Fig.5. Effect of aluminium on the thermal stability of the ovotransferrin fragments. An excess of AlNTA was added to separate solutions of the fragments in 0.5 M Tris–Cl, 0.02 M CO_2, at pH 7.5. The arrows show the position of the endotherm peaks for the apoforms of the fragments. C-fragment, 18.7 mg/ml; N-fragment, 20.4 mg/ml.

63–68°C [2]. However, full stabilization occurs at 50% saturation of the sites, even though ovotransferrin can bind two Al. We have examined the effect of Al on the isolated fragments of ovotransferrin. There is a shift in the denaturation temperature of the N-terminal fragment from 57°C to about 63°C on addition of AlNTA (fig.5). The endotherm for the iron-free C-terminal fragment undergoes a smaller shift to 62°C. Since the stabilization of the N-terminal fragment by Al (as measured by the shift of the endotherms) is substantially greater than that of the C-terminal fragment, it appears likely that stabilization of ovotransferrin by aliminium results from the binding of Al at the N-terminal site.

Acknowledgement

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Note

Reference to a company or product name does not imply approval or recommendation of the product by the US Department of Agriculture to the exclusion of others that may be suitable.

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