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Calorimetric Study of the Rabbit Hepatic Galactoside Binding Protein: Effects of Calcium and Ligands†

Dudley K. Strickland, Thomas T. Andersen,‡ Robert L. Hill, and Francis J. Castellino*

ABSTRACT: Differential scanning calorimetry has been used to examine the thermal denaturation of rabbit hepatic galactoside binding protein. In the absence of Ca^{2+} or ligands, the inactive binding protein shows a single transition with a T_m of 46 ± 0.5 °C and an enthalpy of denaturation of 0.891 cal g^{-1}. In the presence of 20 mM $CaCl_2$, the active binding protein has a single transition with a T_m of 61 °C and an enthalpy of denaturation of 2.67 cal g^{-1}, indicating that Ca^{2+} markedly stabilizes the protein toward thermal denaturation. The T_m values of the binding protein–Ca^{2+} complexes with asialoorosomucoid or lactose are 64 and 63 °C, respectively. The enthalpy of denaturation in the presence of 20 mM lactose is 3.39 cal g^{-1}, indicating that an additional stabilization (~27%) toward denaturation is provided by binding of specific ligands. Furthermore, the differences in the shape of the denaturation profiles in the presence and absence of ligands suggest that ligand binding influences the denaturation process. Calcium binding, however, stabilizes the galactoside binding protein to thermal denaturation to a greater extent than does ligand binding. Thermal denaturation transitions attributable to the A or the B subunits of the binding protein are not observed, suggesting that the two subunits may be structurally similar.

The rabbit galactoside binding protein, discovered by Ashwell and co-workers (Morell et al., 1968, 1971; Hudgin et al., 1974; Kawasaki & Ashwell, 1976), contains two types of polypeptide chains, designated the A (apparent M_r 48 000) and B chains (apparent M_r 40 000), in a molar ratio of one A and two B chains, as determined by sodium dodecyl sulfate gel electrophoresis. The detergent-solubilized rabbit protein appears to have the subunit structure A_2B_4, in the absence of calcium, with a molecular weight of 234 000, as determined by sedimentation equilibrium (Andersen et al., 1981). The binding protein from rat liver has a similar molecular weight (Andersen et al., 1981), although it contains identical subunits (M_r 47 000; Tanabe et al., 1979). The binding proteins from all species, however, require Ca^{2+} for binding activity. Molecular weight studies indicate that the binding protein with a subunit structure A_2B_4 aggregates on binding of Ca^{2+} to give the active binding protein $(A_2B_4)_{2-3}$. Moreover, Ca^{2+} binding is accompanied by quenching of the intrinsic fluorescence of the protein.

† From the Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556 (D.K.S. and F.J.C.), and the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27713 (T.T.A. and R.L.H.). *Received March 12, 1981.* This work was supported by Grants GM-2797 (R.L.H.), HL-19982 (F.J.C.), and HL-05900 (D.K.S.) and a fellowship (T.T.A.) from the Cystic Fibrosis Foundation. D.K.S. is a postdoctoral fellow of the National Institutes of Health.

‡ Present address: Department of Biochemistry, Albany Medical College, Albany, NY 12208.

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Direct binding studies, as well as analysis of fluorescence quenching, indicate that the dissociation constant for the protein–Ca^{2+} complex is in the range of 1–2 mM (Andersen et al., 1981). However, the circular dichroic and ultraviolet absorption spectra of the binding protein indicate that no major changes in the conformation of the protein occur on Ca^{2+} binding (Andersen et al., 1981).

In this investigation, studies on the thermal denaturation of the rabbit binding protein by the method of differential heat capacity scanning calorimetry are reported. This technique provides the opportunity to further examine the effects of Ca^{2+} and ligand binding on the structure of the protein, including the possibility that it contains separate structural domains. The approach has also been used recently to evaluate separate domains of prothrombin (Ploplis et al., 1981) and of fibrinogen (Donovan & Mihalyi, 1974) and to observe conformational transitions in the subfragment-2 region of myosin (Swenson & Ritchie, 1980).

Materials and Methods

Rabbit hepatic galactoside binding protein was purified by affinity chromatography, as previously described (Hudgin et al., 1974; Andersen et al., 1981). Triton X-100 was removed by washing the second affinity column with freshly prepared buffers containing the detergent Brij 58 (Sigma) at concentrations of 1.0 mg mL^{-1}. After elution from the second affinity column, the lectin was shown to be pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and to contain both A (apparent M_r 48 000) and B (apparent M_r 40 000) polypeptide chains in a 1:2 A:B ratio as reported. The binding protein was concentrated by ethanol precipitation (addition of 1.0 volume of −20 °C ethanol and centrifugation at 11 000 rpm for 20 min in a Sorvall SS-34 rotor) and resuspended at a concentration of 5.35 mg mL^{-1} in a buffer containing 0.05 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) pH 7.8, 0.1 M NaCl, and 1 mg/mL Brij 58. The binding protein was then dialyzed extensively against this buffer to ensure compatibility of the sample and buffer in the calorimeter. Recovery of protein from the ethanol precipitation step was quantitative, as measured by ultraviolet absorption spectroscopy, by the Amido Schwartz (Schaffner & Weissmann, 1973) protein assay, and by the method of Lowry et al. (1951), and the ligand binding activity was quantitatively recovered, as measured by the binding assay method A of Hudgin et al. (1974).

Orosomucoid (a gift of Dr. K. Schmid, Boston University) was treated with neuraminidase (Grand Island Biological Co.) to generate asialoorosomucoid, as previously described (Andersen et al., 1981). Asialoorosomucoid was dissolved in the same buffer used with the binding protein at 10.50 mg mL^{-1} and dialyzed extensively. Calcium was added from a stock solution of 0.50 M $CaCl_2 \cdot 2H_2O$ (Fisher) in the same buffer, and lactose (Mallinckrodt) was prepared in buffer at 0.50 M.

Calorimetric measurements were made with a heat capacity calorimeter manufactured by Microcal, Inc., Amherst, MA (Model MC-1). The heat capacity of the sample (0.897 mL) was scanned vs. the appropriate reference solution from ~25 to 85 °C at a rate of 1 °C/min with protein concentrations as indicated in the figure legends. The characteristic melting temperature, T_m, was obtained as the maximum of the transition endotherm, and enthalpies of the conformational transitions were determined by integration of the area under the curves using an Apple II minicomputer with the Graphics Tablet hardware. The base lines before and after the transitions were linearly extrapolated to the T_m, and the area above these lines and bounded by the curve of the endotherm was taken as proportional to the transition enthalpy (Swenson & Ritchie, 1980; Jackson & Brandts, 1970). Melting temperatures were reproducible to ±0.5 °C, and enthalpies were reproducible to ±15%. Calculation of the van't Hoff enthalpies (ΔH_{vH}) was accomplished by integration of the experimental heat capacity, as described by Sturtevant (1974). For the determination of the $\Delta H_{cal}/\Delta H_{vH}$ ratio, the values listed in Table I were employed, using a molecular weight of 234 000 for the binding protein.

Table I: Values of Transition Temperatures and Enthalpies of Denaturation[a]

sample	T_m (°C)	ΔH_{cal} (cal/g)	ΔH_{vH} (kcal/mol)
asialoorosomucoid	67	3.46	
lectin	33		
	46	0.89	102
lectin plus Ca^{2+}	33		
	61	2.67	130
lectin plus Ca^{2+} plus ASOR	33		
	64		
lectin plus Ca^{2+} plus lactose	33		
	63	3.39	149

[a] Calcium and lactose, where indicated, were present at 0.02 M; protein concentrations are in the figure legends. ASOR is asialoorosomucoid.

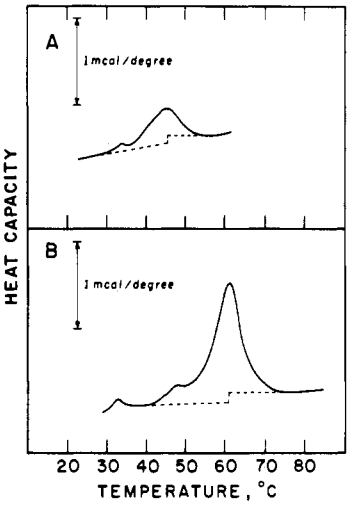

FIGURE 1: DSC scans of rabbit hepatic galactoside binding protein in the absence (A) and presence (B) of 0.02 M $CaCl_2$. The protein concentration was 5.35 mg/mL for (A) and 5.14 mg/mL for (B). In both cases, the scan rate was 60 °C/h. For these experiments, a base line consisting of the appropriate buffer was subtracted from the protein data. Extrapolated base lines are shown as dashed lines, and the area under the curve was determined by integration with an Apple II minicomputer.

Results and Discussion

The thermal denaturation curves for the binding protein with and without Ca^{2+} are shown in Figure 1. A single, major transition was obtained under both conditions. In the absence of Ca^{2+}, the T_m is 46 ± 0.5 °C, and the enthalpy of denaturation is 0.891 cal g^{-1}. In contrast, in the presence of Ca^{2+}, the T_m is 61 ± 0.5 °C, an increase of 15 °C above that found in the absence of Ca^{2+}. The enthalpy increased by 3-fold, to a value of 2.67 cal g^{-1}, in the presence of Ca^{2+}. This corresponds to a value of ΔC_p^d = 0.12 cal deg^{-1} g^{-1}, a fairly large value, but within the range observed for other proteins (Sturtevant, 1977; Privalov & Khechinashvili, 1974).

Minor transitions are observed at 33 ± 0.5 °C for the binding protein in the presence and absence of Ca^{2+}. The basis for this transition is unknown although phospholipids undergo

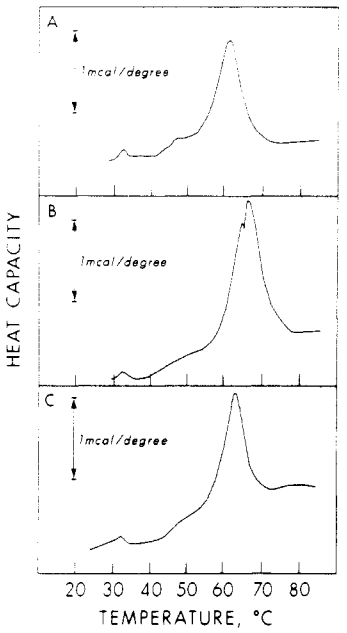

FIGURE 2: DSC scans of the rabbit hepatic galactoside binding protein in the presence of 0.02 M Ca^{2+} (A) plus either asialoorosomucoid (B) or 0.02 M lactose (C). In (A), the lectin concentration was 5.14 mg/mL while in (B) the lectin concentration was 4.28 mg/mL and the asialoorosomucoid concentration was 1.68 mg/mL. In (C), the lectin concentration was 4.90 mg/mL. The scan rate in all cases was 60 °C/h. The samples were scanned vs. buffer and 0.02 M $CaCl_2$ for (A) and (B) and vs. buffer, 0.02 M $CaCl_2$, and 0.02 M lactose for (C). For these experiments, a base line consisting of the appropriate buffer was subtracted from the protein data.

phase transitions in this temperature range. The binding protein is largely free of phospholipid (less than 1 mol/mol; Andersen et al., 1981) although small amounts of preferentially bound detergent cannot be excluded. The presence of a minor transition, seen at 48 °C in the presence of Ca^{2+} (Figure 1B), could reflect binding protein that was not combined with Ca^{2+}, since, as shown in Figure 1A, Ca^{2+}-free protein undergoes a thermal transition at this temperature.

The major structural change in vitro that accompanies activation of the rabbit galactoside binding protein by Ca^{2+} is the aggregation of subunits A_2B_4 to give the fully active species with the subunit structure $(A_2B_4)_{2-3}$. There is little change in the overall conformation of the protein on Ca^{2+} binding as judged by circular dichroic and ultraviolet absorption spectroscopy (Andersen et al., 1981). Only small structural changes are detected on Ca^{2+} binding by fluorescence quenching. Nevertheless, Ca^{2+} binding appears to stabilize the protein toward thermal denaturation, as reflected in a 15 °C increase in the T_m and a 3-fold increase in the enthalpy of denaturation (Table I). It should be noted, however, that the data indicate only that the native lectin–Ca^{2+} complex is more stable relative to the denatured lectin–Ca^{2+} complex than the native lectin, in the absence of Ca^{2+}, relative to its denatured state. This effect could result from either stabilization of the native state or destabilization of the denatured state, or both. These observations suggest that aggregation, induced by Ca^{2+}, contributes to the stabilization of the protein to thermal denaturation. The role that this presumed stabilization plays in the functioning of the lectin in vivo is as yet unknown.

The thermal denaturation curves for the binding protein–Ca^{2+} complex with asialoorosomucoid and with lactose are shown in Figure 2. The curve with asialoorosomucoid as ligand (Figure 2B) reflects the thermal denaturation of both the binding protein and asialoorosomucoid, and the denaturation curve for asialoorosomucoid alone is shown in Figure 3 for comparison. Asialoorosomucoid gives the same curve in the absence or presence of Ca^{2+}, with a $T_m = 67 \pm 0.5$ °C and an enthalpy of 3.46 cal g^{-1} (135 kcal/mol, assuming a M_r of 38 900). These values are comparable to those of a variety of other globular proteins (Privalov & Khechinashvili, 1974). Combination of the binding protein with asialoorosomucoid alters the T_m of the binding protein from 61 to 64 °C whereas the T_m for asialoorosomucoid is changed from 67 to 66 °C. The curves for the complex are also much sharper than those of either protein alone. Moreover, the heat absorbed (14.47 mcal) for denaturation of the complex (Figure 2B) is in close agreement (±6%) with the value calculated from equivalent amounts of each protein measured separately (Figures 1B and 3), *i.e.*, 15.34 mcal. This suggests that binding of asialoorosomucoid does not alter significantly the enthalpy of denaturation of the binding protein.

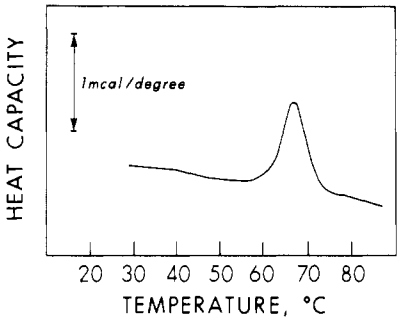

FIGURE 3: DSC scans of asialoorosomucoid. Protein was dissolved at 2.6 mg/mL and scanned at 60 °C/h under constant pressure. The DSC scan of asialoorosomucoid in the presence of Ca^{2+} was similar to the scan shown above. A base line consisting of the appropriate buffer was substracted from the protein data.

Binding of lactose gave similar results to binding with asialoorosomucoid, as shown in Figure 2C. At 20 mM lactose, a concentration that inhibits >95% of the binding protein activity, the T_m for the binding protein increases from 61 to 63 °C whereas the enthalpy increases from 2.67 to 3.39 cal g^{-1}. Again, the sharpening of the curves is observed in the presence of ligand.

Evaluation of the $\Delta H_{cal}/\Delta H_{vH}$ ratio indicates that the nature of the denaturation process of the binding protein, in the presence of calcium and ligand, was different than that for the denaturation of the calcium-free protein. In the absence of calcium, the ΔH_{vH} is 102 kcal/mol with a $\Delta H_{cal}/\Delta H_{vH}$ ratio of 2.0 The presence of calcium increases ΔH_{vH} to 130 kcal/mol with a $\Delta H_{cal}/\Delta H_{vH}$ ratio of 4.8. Addition of lactose to the calcium-protein complex results in a further increase in ΔH_{vH} to a value of 149 kcal/mol and a $\Delta H_{cal}/\Delta H_{vH}$ ratio of 5.3. Since a rigorous interpretation of ΔH_{vH} is only valid for a two-state process, a thermodynamic interpretation of these values in the present case is not possible. However, in complex processes, such as the present case, ΔH_{vH} is a useful measure of the sharpness of the transition (Sturtevant, 1974). The increased ΔH_{vH} values in the presence of calcium and lactose indicate that the transition is sharper and reflect differences in the cooperativity of the denaturation process. One possible intepretation of the increasing $\Delta H_{cal}/\Delta H_{vH}$ values in the presence of calcium and lactose is that these values reflect the size of the cooperative unit undergoing denaturation (Hinz & Sturtevant, 1972; Tsong et al., 1970). This interpretation suggests that the transition involves a number of independent cooperative units, each undergoing a two-state transition. Application of this interpretation to the binding protein would suggest that the size of the cooperative unit decreases as Ca^{2+}

and lactose are added to the system.

Although the binding protein is composed of multiple subunits, a single transition was observed upon thermal denaturation for all forms of the lectin, suggesting that the binding protein is devoid of vastly different independent structural domains, such as those observed in a number of other proteins, e.g., prothrombin (Ploplis et al., 1981), fibrinogen (Donovan & Mihalyi, 1974), and plasminogen (Castellino et al., 1981). It should be pointed out, however, that the large $\Delta H_{cal}/\Delta H_{vH}$ ratios may be consistent with the existence of separate domains undergoing more or less independent denaturation at similar temperatures. These results show that the A and B subunits do not differ markedly in their denaturation parameters, implying that they are structurally similar. This is in accord with their similar amino acid compositions (Kawasaki & Ashwell, 1976). The minor transition, at approximately 46 °C, may well be contributed by dissociated subunits, but this remains to be established.

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Esterification of an Endogenously Synthesized Lipoxygenase Product into Granulocyte Cellular Lipids†

Robert W. Bonser,* Marvin I. Siegel, Sophia M. Chung, Randy T. McConnell, and Pedro Cuatrecasas

ABSTRACT: The human promyelocytic leukemia cell line HL60 can be induced to differentiate into mature granulocytes by exposure to Me_2SO. [1-^{14}C]Arachidonic acid incubated overnight with these cells was incorporated mainly into membrane phospholipids. Stimulation of these cells with the calcium ionophore, A_{23187}, resulted in a rapid release of esterified arachidonic acid from phosphatidylethanolamine and phosphatidylcholine. The released arachidonic acid was metabolized via both the cyclooxygenase and lipoxygenase pathways into three major hydroxylated products, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), 5(*S*)-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE), and 5(*S*),12(*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid (leukotriene B). Arachidonic acid was also incorporated into triacylglycerols and phosphatidylinositol. The lipoxygenase product, 5-HETE, was rapidly esterified into cellular lipids. Thirty minutes after ionophore stimulation, 55% of the total 5-HETE synthesized was esterified into phopholipids and 35% incorporated into acylglycerols. In contrast, the other hydroxylated derivatives of arachidonic acid (HHT and leukotriene B) were not incorporated into acylglycerols or phospholipids. Esterification of hydroxylated metabolites of arachidonic acid into membrane phospholipids may serve to regulate a number of granulocyte functions.

Arachidonic acid metabolism in granulocytes has been the subject of extensive research recently. An activatable phospholipase, which releases arachidonic acid from membrane phospholipids, has been described in neutrophils (Stenson & Parker, 1979a; Waite et al., 1979), and these cells are known to metabolize free arachidonic acid via the cyclooxygenase and lipoxygenase pathways (Goldstein et al., 1978; Borgeat & Samuelsson, 1979a; Goetzl & Sun, 1979; Siegel et al., 1980). The lipoxygenase products have been shown to be chemotactic for neutrophils (Tainer et al., 1975; Turner et al., 1976; Goetzl et al., 1977) and to induce degranulation of specific granules (Stenson & Parker, 1980). Furthermore, arachidonic acid and its metabolites have been implicated in a number of other physiological functions of the neutrophil. These include aggregation and degranulation (Ford-Hutchinson et al, 1979; O'Flaherty et al., 1979; Naccache et al., 1979), the chemotactic process (Hirata et al., 1979), changes in the permeability of the plasma membrane to calcium (Volpi et al., 1980),

† From the Molecular Biology Department, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709. *Received March 16, 1981.*

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