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Increase in the Stability of Avidin Produced by Binding of Biotin. A Differential Scanning Calorimetric Study of Denaturation by Heat†

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ABSTRACT: Transition temperatures (T_{tr}) and enthalpies of denaturation (ΔH_{tr}) for avidin and the avidin–biotin complex were obtained by differential scanning calorimetry. Measurements near pH 7 and 9 indicated little effect of pH, ionic strength, or buffer ion on T_{tr} or ΔH_{tr}. Large increases in both T_{tr} and ΔH_{tr} resulted when avidin bound biotin. T_{tr} increased from 85 to 132°. The increase in ΔH_{tr}, from 300 to 1000 kcal per mol of avidin tetramer, appears to be due primarily to a large increase in heat capacity of the protein upon irreversible denaturation.

The stability of avidin and its complex with biotin has been the subject of several investigations (Green, 1966; Wei and Wright, 1964) since binding of biotin by avidin prevents assimilation of this vitamin (Eakin *et al.*, 1940, 1941; György *et al.*, 1941). The dissociation constant of the complex has been reported to be 10^{-15} M (Green, 1963a). Each of four biotin molecules is bound to one subunit of the avidin tetramer, and the binding appears to be noncooperative (Green, 1966). Wei and Wright (1964) have found that although avidin is heat labile at 100°, the complex is stable at that temperature—indeed, autoclave heating at 120° for 15 min was required to release bound biotin. Avidin and its complex with biotin are stable in 9 M urea, and over a pH range from 2 to 13.7 (Green, 1963b). A concentration of 7 M guanidine hydrochloride, just sufficient to denature avidin, does not denature the avidin–biotin complex (Green, 1963b). However, the denaturation temperatures of avidin and the avidin–biotin complex and the enthalpies of denaturation have never been determined.

We have employed the method of differential scanning calorimetry (DSC) to quantitatively evaluate the heat stability of avidin and the avidin–biotin complex. We carried out these studies at pH 9 and 7, since egg white and whole egg normally are at these pH values when they are heat processed in commerce (*e.g.*, during pasteurization or drying). Ample evidence exists to demonstrate the usefulness of DSC for the study of thermal denaturation of proteins (Beck *et al.*, 1965; Delben *et al.*, 1969; Tsong *et al.*, 1970; Jackson and Brandts, 1970; Crescenzi and Delben, 1971) as heats of transition can be quantitated more easily by DSC than by the older differential thermal analysis technique (Steim, 1965). Hermetically sealed containers which will withstand internal pressures of several atmospheres make possible determination of transition temperatures above the boiling point of water, such as those we have observed for the denaturation of the avidin–biotin complex.

Experimental Section[1]

Materials. Avidin was prepared by a variation of the method of Green and Toms (1970). Difference absorbance assay (Green, 1963a) yielded a value of 15.0 μg of biotin bound per mg of this preparation of avidin. The *d*-biotin was Calbiochem lot 000920 used without further purification. Ribonuclease was Worthington Biochemical Corp. lot R7LE. Lysozyme was Difco Labs. lot 0465-10. Tris(hydroxymethyl)-methylaminopropanesulfonic acid (Taps)[2] buffer was Sigma Chemical Co. lot 99B-5230. Pure indium was supplied by the Instrument Products Division of E. I. duPont de Nemours & Co. Benzoic acid was a National Bureau of Standards standard sample. The other substances used for calibration of the differential scanning calorimeter, the purest commercial material locally available, gave thermograms indicative of high purity.

Methods. Protein solutions used for quantitative measurement of denaturation are listed. Protein solutions at other concentrations were used in preliminary experiments to determine transition temperatures: for avidin, (a) 73.5 mg/ml at pH 6.84 and (b) 16.4 mg/ml at pH 6.62 in 0.1 M KCl–0.02 M in potassium phosphate; (c) 14.6 mg/ml at pH 9.04 in 0.1 M KCl–0.02 M borate; (d) 35.9 mg/ml at pH 9.14 in 0.1 M KCl plus universal buffer at half the concentration specified by Britton and Welford (1937). For avidin plus biotin, a stoichiometric excess of biotin (crystals) was added to an aliquot of each of a–d. The amount of biotin added exceeded its solubility at room temperature, so the solutions were drawn

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

[2] Abbreviation used is: Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid.

off from the crystals for denaturation measurements of the avidin–biotin complex; (e) ribonuclease, pH 3.91: 49.8 mg/ml in 0.1 M KCl; (f) ribonuclease, pH 6.84: 51.0 mg/ml in 0.1 M KCl, 0.02 M in potassium phosphate; (g) lysozyme, pH 8.96: 16.8 mg/ml in 0.1 M Taps, adjusted to pH 8.96 with 0.1 M KOH. Protein concentrations were calculated from absorbance measured with a Cary 15 spectrophotometer. The following optical factors ($E^{1\%}_{1\,cm}$) were used: avidin, 15.4 at 281 mμ (Green and Toms, 1970); ribonuclease, 7.38 at 277 mμ (Scott and Scheraga, 1963); lysozyme, 26.3 at 281 mμ (Sophianopoulos *et al.*, 1962). Molecular weights used for calculations were: avidin tetramer, 64,800 (Green and Toms, 1970); ribonuclease, 13,683 (Hirs *et al.*, 1956); lysozyme, 14,400 (Sophianopoulos *et al.*, 1962).

Measurements of pH were made at room temperature with a Radiometer Model 26 pH meter. The change in pH of phosphate buffer solution is less than one-tenth of a pH unit between 25 and 95° (Bates, 1964). The known temperature dependence of universal buffer solution (Britton and Welford, 1937) indicates a pH of 8.6 at the denaturation temperature of avidin, 85°. At 132°, the denaturation temperature of the avidin–biotin complex, the pH value of this buffer is probably not more than a few tenths of a pH unit different from its pH value at 85°. The pH of the borate buffer also drops several tenths of a pH unit between room temperature and 132° (Bates, 1964). We estimate the pH of this buffer to be about 8.7 at 132°.

Because Wei and Wright (1964) have noted that increase in ionic strength increased the stability of the avidin–biotin complex, calorimeter runs were also carried out using solutions of avidin and avidin plus biotin adjusted to ionic strength 0.3 by addition of solid KCl. No changes in ΔH_{tr} or denaturation temperatures were observed.

Differential scanning calorimeter thermograms were recorded on a Du Pont Model 990 thermal analyzer with two different DSC cells. Melting point determinations from thermograms of indium and benzoic acid indicated that the thermocouple which measured sample temperature was correct to within 0.5° for cell 1. Cell 2 required a temperature correction which was a function of temperature. A temperature calibration curve was obtained for this cell from the observed melting points of indium, benzoic acid, α-naphthol, naphthalene, *p*-dichlorobenzene, and water ice. With the temperature correction, the reported temperatures are also accurate to 0.5°. Samples of 20 μl of protein solutions were pressure sealed in Du Pont coated aluminum hermetic pans, which weigh approximately 58 mg. The reference material was either a sealed sample pan containing water or a flat piece of metal of appropriate heat capacity. For some early runs, the cell was purged with nitrogen gas at 10 ml/min. This procedure was discontinued for later runs since no improvement in signal-to-noise ratio or in linearity of base lines was apparent. Following the advice of Dr. Paul Levy of Du Pont Instruments, we calculated the calibration coefficient, E, for cell 1 from the thermograms of weighed amounts of materials with known enthalpies of fusion. The coefficient was plotted as a function of temperature for five temperatures: 57° (myristic acid), 67° (azobenzene), 80° (naphthalene), 122° (benzoic acid), and 157° (indium). From this curve, the appropriate value of E for each protein thermogram was interpolated at the temperature of the maximum deflection observed in that thermogram. For cell 1, E was 0.837 at 85° and 0.920 at 132°. The calibration data for cell 2 were obtained from the melting thermograms for indium, pentachlorophenol, benzoic acid, naphthalene, biphenyl, and water ice. The calibration coefficient determined from heat capacity measurements of a weighed sapphire standard over the range 45–140° was essentially identical. For cell 2, E was 0.989 at 85° and 1.052 at 132°. The estimated error in determining E is ±2%.

Since measurements of the heat capacities and heats of fusion of solids are not really equivalent to the measurement of the enthalpy of denaturation of a protein in solution, we have also determined the enthalpies of denaturation of ribonuclease and lysozyme to check our instrument calibration for gross errors. The enthalpies of denaturation we obtained for ribonuclease appear to be the same, within experimental error, as those reported by Tsong *et al.* (1970), who used a much more sophisticated calorimeter.

The onset of denaturation was relatively insensitive to changes in programming rate, but the temperature of maximum deflection ("peak") was shifted upward with increased programming rate. This occurs because the rate of denaturation was comparable to the programming rate. Denaturation thermograms of ribonuclease were determined at programming rates of 12.4, 6.2, 2.5, and 1.2° per min. The temperature of maximum deflection was extrapolated to a programming rate of 0°/min. The extrapolated temperature, 64°, agreed with the published value of 64° for pH 6.83 (Hermans and Scheraga, 1961). On the basis of this extrapolation, and extrapolations of T_{tr} of other proteins as a function of program rate, we have determined a temperature correction of −6° is applicable to the 12.4°/min thermograms, and of −1° to the 2.5°/min thermograms when comparisons are made between the peak of the DSC thermogram and values of T_{tr} reported in the literature. For convenience in describing the denaturation of avidin, we report values of T_{tr} calculated as described above. However, since the denaturation is irreversible, no real T_{tr} exists. This is discussed further below.

Replicate samples of each protein were run in cell 1 at two programming rates—12.4°/min to give a large peak area for integration to determine the heat of transition, and at 2.5°/min to give a closer approach to the "denaturation temperature," T_{tr}. Cell 2 was programmed only at 10.0°/min. Temperatures were reproducible to better than 1°, and areas of replicate scans, measured with a planimeter, were reproducible within about 2%. Equation 1 was used to calculate

$$\Delta H_{tr}\ (\text{mcal/mg}) = \frac{\text{area (in.}^2\text{)}}{\text{weight (mg)}} \{\text{time base (sec/in.)} \times E \times \text{sensitivity [mcal/(sec in.)]}\} \quad (1)$$

enthalpy of denaturation (ΔH_{tr}) from peak area.

To determine the peak area, a straight line was drawn under the thermogram connecting the base line at the temperature of initial deflection with the base line at the temperature at which the heat flow due to denaturation appeared to cease. The area enclosed by this straight line and the thermogram was used to calculate the enthalpy of denaturation.

Avidin–biotin solutions were held at fixed elevated temperatures to determine if the complex could be denatured if sufficient time were allowed at temperatures considerably lower than T_{tr}. Samples in sealed pans were heated to 98 or 120° in program mode, then held at these temperatures for 15 min. The samples were cooled about 50° by cooling the entire heating assembly of the DSC cell. The samples were then reheated in programming mode to observe whether an endotherm occurred at the characteristic T_{tr}. When alkaline buffer solutions (universal buffer or ammonium carbonate)

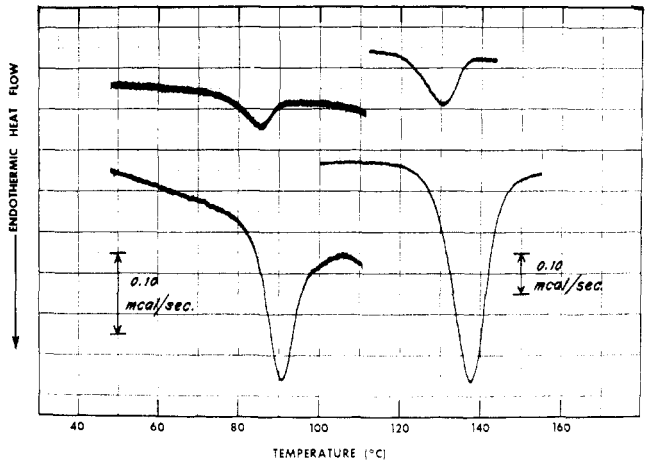

FIGURE 1: Denaturation thermograms of pH 6.84 solutions of avidin with (right) and without (left) a stoichiometric excess of biotin, determined in DSC cell 1. Protein concentration was 73.5 mg/ml; heating rate, 2.5 deg min^{-1} (upper curves) and 12.5 deg min^{-1} (lower curves). Nominal sensitivity of instrument was 0.05 mcal sec^{-1} $in.^{-1}$ for curves on left, 0.10 mcal sec^{-1} $in.^{-1}$ for curves on right (see scales on figure).

were used, the results of these experiments could not be interpreted, perhaps because of reaction of the buffer components with the sample pan. For example, reheating buffer alone or biotin in buffer gave abnormal thermograms—exotherms at nonreproducible temperatures and irregular base lines. However, experiments in phosphate buffer at pH 6.84 gave no such difficulties, and results of these experiments are reported below.

The heat capacity difference between denatured and native avidin, ΔC_p, was required to interpret the large difference observed between the enthalpies of denaturation obtained for avidin and avidin plus biotin (see below). The accuracy of this measurement was limited by the sensitivity of the instrument, and a protein solution of high concentration was required. A 3-mg sample of avidin in 10 μl of solvent was programmed through its transition temperature at 12.4°/min, cooled, and reprogrammed identically. The ΔC_p between 25 and 70° was calculated at 2.5° intervals from the vertical separation of the traces. The calibration coefficient of the instrument, the programming rate, and the exact weight of protein used enter into this calculation. The 19 values of ΔC_p so calculated were fit to unweighted linear and quadratic least-squares equations by means of Trautman's (1969) programs PI-1033 and PI-1058 for the Olivetti P-101. Estimates of ΔC_p were also obtained from the vertical displacement of the base line between the beginning and the end of denaturation. An illustration of this technique is clearly presented in Figure 2 of the paper by Jackson and Brandts (1970).

Attempts were made to observe denaturation of both avidin and the avidin–biotin complex by optical rotation, using apparatus and methods described previously (Donovan *et al.*, 1969). Solutions of avidin at pH 6.8 and 9.1 became turbid at 70° without significant change in rotation, so that denaturation could not be followed by this technique. Solutions of avidin plus biotin remained clear to above 95° without significant change in optical rotation.

The rate constant for denaturation was calculated at various temperatures and heating rates according to the method of Beech (1969). The deflection from the base line, at any temperature, is proportional to the rate of heat flow into the sample, dH/dt, and thus is a measure of the rate of denaturation. The relative amount of native protein present at any temperature can be determined by measuring the fractional area under the peak above that temperature. The fraction of the area below that temperature, designated a, is proportional to the relative amount of protein already heat denatured. If A represents the total peak area, then the rate constant for denaturation at temperature T is given by

$$k = (\text{heating rate})(dH/dt)/(A - a) \qquad (2)$$

First-order kinetics of denaturation are assumed. The calibration coefficient, E, of the cell does not enter the calculations.

TABLE I: Enthalpies and Temperatures of Transition.

Protein	pH[a]	T_{tr}[b] (°C)	ΔH_{tr} (kcal/mol)[c]
Ribonuclease[d]	6.84	64	91 ± 5
	3.91[e]	60	67 ± 5
Lysozyme	8.96	62	127 ± 10[f]
Avidin	6.62	85	308 ± 12
	6.84	85	298 ± 10
	9.04	85	265 ± 20
	9.14	85	288 ± 15
Avidin plus biotin	6.62	132	1108 ± 55
	6.84	131	1065 ± 30
	9.04	133	1014 ± 50
	9.14	132	939 ± 30

[a] Measured at room temperature. See Methods for solution conditions. [b] Temperatures have an expected error of ±0.5°, and have been corrected for programming rate (see text). [c] Errors listed are maximum expected errors (Steinbach and King, 1950) and include errors in sample preparation, uncertainty in calibration coefficient and reproducibility of (area)/(programming rate) at different programming rates. [d] See Lumry *et al.* (1966) and Tsong *et al.* (1971) for the dependence of ΔH_{tr} on temperature and pH, and a summary of earlier experiments carried out by other techniques. Values of ΔH_{tr} reported here agree with those defined by Tsong *et al.* (1971) as $\Delta H_{cal}(25°)$. [e] Unbuffered solution in 0.1 M KCl. [f] Crescenzi and Delben (1971) report a ΔH_{tr} of 138 ± 7 at pH 5.4.

Results

Figure 1 shows sample thermograms for avidin and avidin plus biotin determined with DSC cell 1. The areas under the peaks in Figure 1, after correction for the small temperature variation in the calibration coefficient, E, are proportional to the enthalpies of denaturation (eq 1). Values of ΔH_{tr} obtained for ribonuclease, lysozyme, avidin, and avidin plus biotin by use of eq 1 are given in Table I.

Some avidin runs were continued to 150°. No endothermic reaction near 130° was observed, so the avidin sample did not contain any avidin–biotin complex. This result agrees with the high purity obtained on assay. Several runs of buffer solutions which were saturated with biotin at room temperature showed no endotherms between 20 and 160°. Accord-

ingly, the endotherm at 85° was assumed to be a measure of the enthalpy of denaturation of avidin exclusively, and the endotherm near 130° was attributed solely to denaturation of the avidin–biotin complex. A 100% conversion of avidin to avidin–biotin complex was assumed, since no peak at 85° was observable on the thermograms of the avidin plus biotin runs. Repeat runs of denatured samples of avidin and of avidin plus biotin produced no endotherms, indicating that both species are denatured irreversibly.

Holding of pH 6.84 solutions of avidin plus biotin at 98° or at 120° for periods of 15 min (as described in Methods) had no effect on the size of the endotherm observed at T_{tr}.

The data for the heat capacity difference between native and denatured avidin in the temperature range 25–70° were adequately fit by

$$\Delta C_p \text{ (kcal mol}^{-1} \text{ deg}^{-1}) = 5.99 - 0.048t + 0.00118t^2 \quad (3)$$

or, alternatively

$$\Delta C_p \text{ (kcal mol}^{-1} \text{ deg}^{-1}) = 3.56 + 0.0633t \quad (4)$$

where t is temperature in degrees centigrade. A test for significance showed that the quadratic coefficient of eq 3 and the linear coefficient of eq 4 both exceeded the product of the standard error of the coefficient with the Student's t value at the 1% probability level (16 and 17 degrees of freedom, respectively).

As stated above, denaturation thermograms were also used to estimate ΔC_p. The displacement of the base line from the beginning to the end of the transition was consistent with a large ΔC_p. As presently employed, this apparatus cannot provide reliable values of ΔC_p from base line displacements; the calculation lacks both precision and accuracy. For avidin, ΔC_p was calculated from base-line displacements to average 12 kcal mol^{-1} deg^{-1} at 85° (range 9.4–13.8). For avidin plus biotin, the average ΔC_p was 12.4 kcal mol^{-1} deg^{-1} at 132° (range 10.5–14.3).

The temperature dependence of the rate constant for thermal denaturation of avidin at pH 6.84 is given in Figure 2. The rate constants obtained at two heating rates which differ by a factor of five are fit fairly accurately by a single straight line. Accordingly, gross instrumental errors, such as a thermal lag producing a difference in temperature between the sample thermocouple and that of the protein solution, appear to be absent. The activation energy and entropy calculated from this figure are 90 kcal mol^{-1} and 270 EU.

It is evident from Figure 2 that a value of T_{tr} cannot be given for an irreversible denaturation. However, since it is convenient to have a single number characteristic of the temperature range of the transition, we give in Table I an estimate of the temperature at which the peak in the thermogram would be observed if a very slow heating rate were employed.

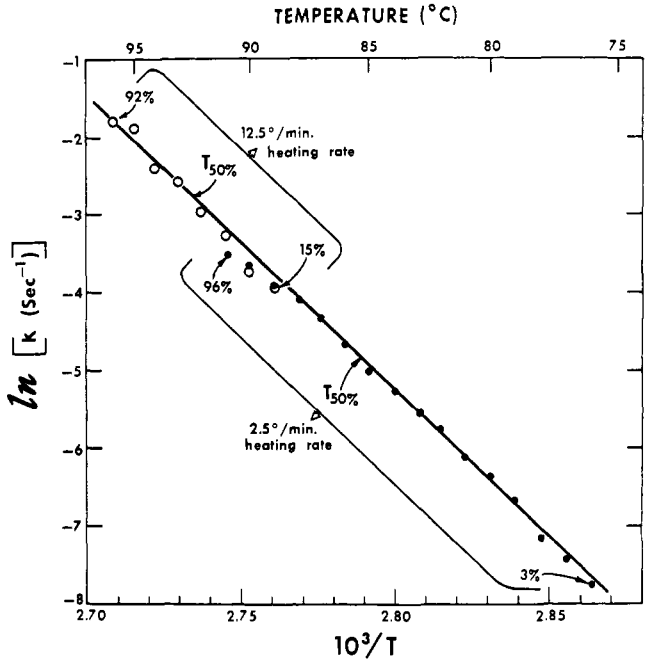

FIGURE 2: Arrhenius plot for thermal denaturation of avidin, pH 6.84. The per cent of denaturation is indicated for points at the temperature extremes of each of two runs made at different heating rates. The temperatures at which 50% of the protein is denatured are also indicated.

Discussion

Clearly, *the binding of biotin greatly increases the stability of avidin.* The *increase of 46–47° in its transition temperature* is substantial. Binding of substrates or substrate analogs generally increases the heat stability of proteins, but the increases in stability which have been reported are not as great as we have observed for avidin. For example, when ferric and bicarbonate ions are bound to conalbumin, a rate of heat denaturation equivalent to that observed for untreated conalbumin requires a temperature increase of about 15° (Azari and Feeney, 1958). Since the denaturation of the avidin–biotin complex is observed at 131–132° in containers sealed at room temperature, we have considered whether the internal pressure of several atmospheres affects the denaturation temperature. Gill and Glogovsky (1965) have shown that 680 atm of pressure *lowers* the temperature of denaturation of ribonuclease by about 2°. Avidin should be comparable to ribonuclease in its pressure dependence. The molecular weights of ribonuclease and the avidin subunit differ by only about 20%, both are globular proteins with approximately equal proportions of charged groups, and have similar hydrophobicities (Bigelow, 1967). Thus, the effect of a few atmospheres of pressure should not only be small, but in the direction of lowering the transition temperature of avidin, not of raising it. In this regard, it should also be noted that ΔC_p discussed here is really not a true ΔC_p because of the pressure change with temperature in the hermetically sealed sample pans.

In the absence of biotin, the enthalpy of denaturation of avidin is comparable to that of other globular proteins. Specific enthalpies of denaturation are 4.8–6.5 cal/g for ribonuclease (Table I), 8.8 cal/g for lysozyme (Table I), and 3.7–5.7 cal/g for chymotrypsinogen (Jackson and Brandts, 1970, Biltonen *et al.*, 1971) compared to 4.6 cal/g for avidin. But the increase in enthalpy of denaturation observed on addition of biotin is remarkable—*binding of biotin more than triples the enthalpy of denaturation of avidin.* The measured enthalpies of denaturation of the avidin–biotin complex (Table I) range from 14.4 to 16.4 cal per g (approximately 1000 kcal/mol of avidin tetramer). This enthalpy of denaturation greatly exceeds those so far reported for any other protein.

A significant contribution to the large increase in enthalpy of denaturation might be attributable to the enthalpy of binding of biotin to avidin. Suurkuusk and Wadsö (1972)

have recently determined that the enthalpy of binding 4 mol of biotin/mol of avidin tetramer is −90.0 kcal at 25.0°. This result is similar to that reported earlier by Green (1966). Suurkuusk and Wadsö have shown that the temperature dependence of the enthalpy of binding of biotin to avidin is linear over the temperature range they investigated, 25–43°, and that ΔC_p of binding is −0.95 kcal/mol of avidin tetramer deg^{-1}. A linear extrapolation of their data gives −186 kcal for the enthalpy of binding 4 mol of biotin/avidin tetramer at 132°. If we make the natural assumption that the binding site of avidin is destroyed on heat denaturation, then the enthalpy of denaturation of avidin plus biotin should then be about 186 kcal greater than that of avidin alone. Since the enthalpy of denaturation of the avidin–biotin complex is approximately 700 kcal mol^{-1} greater than that of avidin, it is evident that the enthalpy of binding will only account for about one-fourth of the observed increase in enthalpy of denaturation.

At least two possibilities remain to explain the large increase in enthalpy of denaturation. Perhaps upon denaturation of avidin at 85°, no dissociation of the tetramer occurs, but dissociation into subunits does take place upon denaturation of the avidin–biotin complex at 132°. Then the "excess enthalpy" might be attributable to the enthalpy of separation of denatured subunits of the avidin–biotin complex. We feel that this possibility is improbable, particularly since some experimental evidence for an alternative explanation exists.

It seems likely that the large difference in the enthalpies of denaturation is related to the large difference in the temperatures of denaturation of avidin and of the avidin–biotin complex. If the heat capacity difference between native and denatured avidin (with or without bound biotin) was sufficiently large, it would produce a substantial contribution to ΔH_{tr} at the higher temperature. As described above, we have obtained, by two different methods, estimates of ΔC_p which agree reasonably well. Equations 3 and 4 give comparable values for ΔC_p in the 25–70° temperature range. However, upon extrapolation beyond this range, they differ significantly in their predictions of ΔC_p. At 85 and 132°, respectively, eq 3 gives ΔC_p values of 10 and 19 kcal mol^{-1} deg^{-1}, while eq 4 gives 8.9 and 11.8 kcal mol^{-1} deg^{-1}. The latter values are in better agreement with estimates of ΔC_p obtained from baseline displacement, 12–12.4 kcal mol^{-1} deg^{-1} at 85 and 132° (see above). These values of ΔC_p are sufficiently large to account for the observed 700 kcal mol^{-1} difference in enthalpies of denaturation. There are only a few values of ΔC_p reported for proteins with which comparisons can be made. Jackson and Brandts (1970) and Biltonen *et al.* (1971) report ΔC_p values of 2.8–4.2 kcal mol^{-1} deg^{-1} for chymotrypsinogen (mol wt 25,700) in the temperature range 25–50°, and Forrest and Sturtevant (1960) report a ΔC_p of 9.9 kcal mol^{-1} deg^{-1} for the reversible acid denaturation of ferrihemoglobin (mol wt 68,000) at 20°.

Integration of eq 4 between 85 and 132° gives a $\Delta(\Delta H_{tr})$ of 467 kcal/mol of avidin tetramer. Integration of a (constant) average ΔC_p of 12.2 kcal mol^{-1} deg^{-1} over the same temperature range yields a $\Delta(\Delta H_{tr})$ of 573 kcal mol^{-1}. When the calculated ΔH of 186 kcal mol^{-1} for binding of biotin at 132° is added, total values of $\Delta(\Delta H_{tr})$ of 653 and 759 kcal mol^{-1} are obtained for the difference between the enthalpies of denaturation of avidin and of the avidin–biotin complex. Considering the uncertainty in our estimates of ΔC_p, we consider this satisfactory agreement with the experimental enthalpy differences which range from 640 ± 50 to 800 ± 70 kcal mol^{-1} (see Table I).

Both eq 3 and 4 predict that ΔC_p increases with temperature. Equation 3 predicts a marked increase in ΔC_p with temperature above 85°. Estimates from base-line displacements at 85 and 132° (see above) indicate the ΔC_p may be approximately constant with temperature. Both Jackson and Brandts (1970) and Biltonen *et al.* (1971) have observed that the ΔC_p for chymotrypsinogen decreases with temperature. Although avidin may differ from chymotrypsinogen because it contains subunits, there are not enough values of ΔC_p reported for proteins to make any generalizations about the expected temperature dependence of ΔC_p at this time.

Aggregation. The turbidity observed in heated solutions of avidin during attempts to measure the denaturation of avidin by optical rotation suggests that aggregation of denatured protein might occur in the temperature range of the transition. Several aspects of the experimental data suggest that aggregation is not an important factor in the calorimetric experiments. (1) Turbidity was observed to begin at about 70° in the optical rotation experiments yet no deflection was observed below 75° on any thermogram. (2) Thermograms obtained at heating rates which differ by a factor of five give the same enthalpy of denaturation, within experimental error. (3) Rate constants for melting, calculated from fractional areas under the melting curve, as described in the Experimental Section, are the same when obtained from these thermograms at the two different heating rates (Figure 2). (4) At the upper end of the transition range, when most of the protein is denatured and the thermograms are most likely to show heat effects due to aggregation, the plot of ln k *vs.* $1/T$ (Figure 2) does not show curvature. (5) Aggregation should be greater at higher protein concentration. Over the concentration range of 15–74 mg/ml (a factor of five in concentration) no effect of concentration on ΔH_{tr} is observed. These results suggest that heat effects due to aggregation are absent.

Acknowledgments

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A Carbon-13 Nuclear Magnetic Resonance Study of Nucleotide–Metal Interactions. Binding of Manganese(II) with Adenine Nucleotides†

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ABSTRACT: The influence of paramagnetic Mn^{2+} ions on the proton decoupled ^{13}C nuclear magnetic resonance spectra of adenosine monophosphates has been observed. The C-5 and C-8 resonances are broadened preferentially to the C-2, C-4 and C-6 resonances by Mn^{2+} ions in 5′-AMP, 3′-AMP, and 2′-AMP. The five ribose carbon resonances for the three nucleotides are little affected. The metal ion is therefore held near the N-7 position of the base. The present data indicate that specific line-broadening effects are observed in ^{13}C spectra which can be used in determining the nature of the paramagnetic ion binding site. The line-broadening behavior is similar for the three adenosine mononucleotides. A possible model for the Mn^{2+} complex with the nucleotides is discussed.

Metal ions play an important role in protein chemistry as well as in nucleic acid processes. Numerous investigations have been carried out in studies of the nature of the metal complexes. The recent advances in metal interactions with nucleosides, nucleotides, and related compounds have been summarized (Izatt *et al.*, 1971; Phillips, 1966; Weser, 1968). Nuclear magnetic resonance is one of the powerful techniques that is used in the determination of metal binding sites and the nature of the complex in these systems.

^{31}P nuclear magnetic resonance (nmr) showed that Mn^{2+} and Co^{2+} ions bind to the phosphate group of 5′-AMP (Shulman *et al.*, 1965). The Mn^{2+} ion has been shown using ^{1}H line-broadening experiments of the H-2 and H-8 resonances to affect the H-8 resonance of 5′-AMP most strongly (Chan and Nelson, 1969). Water proton T_2 relaxation time studies showed that 5′-AMP and 2′-AMP have one binding site for the Mn^{2+} ion (Heller *et al.*, 1970).

In this paper we wish to illustrate the potential and the applicability of the ^{13}C nmr approach in the study of adenosine monophosphates (2′-AMP, 3′-AMP, 5′-AMP) in D_2O solutions containing small quantities of Mn^{2+} ions. The ^{13}C nmr spectrum of AMP consists of ten lines, five arising from the base and five from the ribose moiety of the molecules. Therefore with ten nuclear probes, we can expect to obtain detailed information about the nature of the metal binding site. We use an approach that has been extensively applied and involves the measurement of the broadening of specific lines in a spectrum upon the addition of paramagnetic ions. The influence of paramagnetic ions on nuclear relaxation times and hence on line broadening has been analyzed by Solomon (1955), Bloembergen and Morgan (1961), Bernheim *et al.* (1959), and reviewed by Eaton and Phillips (1965). The binding of the paramagnetic ion leads to broadening of the ^{13}C resonances. When the electron-spin, nuclear-spin, dipole–dipole interaction is the predominant relaxation mechanism, then $T_1 = T_2$ and the relaxation times are extremely

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