Differential Scanning Calorimetry of Apo-, Apophosphoryl, and Metalloalkaline Phosphatases

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(Received for publication, April 5, 1977)

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Differential scanning calorimetry has been applied to assess the nature of the thermally induced denaturation of the metal-free apo and apophosphoryl derivatives of the dimeric Zn⁺ metalloenzyme alkaline phosphatase (Escherichia coli). The transitions of both species deviate markedly from the two-state model, giving rise to detectable intermediates on renaturation as detected on repetitive calorimetric scans. Consistent with the temperature, enthalpy, and cooperativity of the observed transitions the reversible denaturation of both apoproteins can be described as a four-state process \( N = N' = N'' = U \) and the thermodynamic parameters characterizing the native and intermediate states \((N, N', N'')\) relative to the unfolded state \(U\) have been determined. Thus for apoprotein phosphatase at \(30^\circ\), \(\Delta G_c = 19.9\) kcal mol\(^{-1}\) and \(\Delta G_n = 2.3\) kcal mol\(^{-1}\) while for apophosphoryl alkaline phosphatase \(\Delta G_c = 5.6\) kcal mol\(^{-1}\), \(\Delta G_n = 2.4\) kcal mol\(^{-1}\). The destabilization of the native state of the apoprotein arising as a consequence of specific phosphorylation at a single serine residue is compatible with the postulated subunit interactions (negative cooperativity) documented for this enzyme.

The binding of the native metal ion Zn⁺ at the two active center binding sites increases the stability of the enzyme by \(-70\) kcal mol\(^{-1}\). The binding of the first two Zn⁺ ions appears to be cooperative. Successive addition of a second pair of Zn⁺ ions and 1 eq of Mg⁺ further stabilizes the metalloenzyme by 30 and 10 kcal mol\(^{-1}\). Metal ion binding does not, however, markedly increase the cooperativity of the observed transitions. Determination of the entropic contribution to the relative free energy of the metalloproteins indicates that metal ion association results in the generation of structures of high internal order relative to the apoenzymes.

The application of differential scanning microcalorimetry (1–3) to the investigation of thermally induced conformational transitions of proteins has allowed a rigorous assessment of the thermodynamic parameters describing the unfolding process (4–6). These results have led to an understanding of the nature and magnitude of the forces contributing to the stabilization of the folded polypeptide at ambient temperatures (7, 8). To date these studies have been performed on a variety of monomeric proteins for which detailed structural information is available. The ability of the dimeric Zn⁺ metalloenzyme, alkaline phosphatase, and the metal-free apoenzyme to undergo reversible dissociation and unfolding render it a suitable protein for the utilization of scanning calorimetry as a probe of enzyme secondary, tertiary, and quaternary structure.

Alkaline phosphatase (EC 3.1.3.1) from Escherichia coli is a dimer \(M_r = 86,000\) composed of identical subunits symmetrically disposed about a 2-fold rotation axis \((9–11)\). The enzyme is a nonspecific phosphate monoester hydrolase with two separate active centers/dimer. In the native state, the protein contains four Zn⁺⁺ ions and one to two Mg⁺⁺ ions \((12)\). The metal ions can be reversibly removed from the protein with concomitant loss of catalytic function which can be completely restored on addition of 2 eq of Zn⁺⁺ ion/dimer \((13–16)\). The native enzyme possesses a remarkable thermostability and is relatively insensitive to hydrogen ion concentration, displaying only minor structural perturbations (as monitored by optical spectroscopy) between pH 4 and pH 8 \((9, 17)\). Removal of the intrinsic metal ions at neutral pH does not result in dissociation of the protein dimer \((18, 19)\) and only minor perturbations in secondary structure are detected \((19, 20)\).

The importance of the metal ions in maintaining the conformational integrity of the enzyme and its resistance to thermally induced structural transitions has been suggested \((21, 22)\), but little experimental evidence has been obtained documenting these proposals.

The mechanism of action of alkaline phosphatase involves the intermediate phosphorylation of the enzyme at a unique serine residue \((23, 24)\), a process subject to allosteric regulation as evidenced by the existence of negative homotropic interactions between the subunits \((25–27)\). Under certain conditions apophosphoryl enzyme containing 1 phosphoserine residue/enzyme dimer can be isolated \((28)\). The symmetrical relation of the subunits is altered in the phosphorylated apoenzyme \((27)\). Differential scanning calorimetry applied to alkaline phosphatase provides a sensitive method for: (a) comparing the nature of thermally induced structural changes of a dimeric enzyme to those of the monomeric proteins previously studied; (b) assessing the consequences of disruption of the symmetric
disposition of the subunits on covalent phosphorylation; (c) determining the role of the metal ions in stabilizing protein structure; and (d) probing the proposed structural perturbations induced by variation of the hydrogen ion concentration.

MATERIALS AND METHODS

Enzyme and Chemicals — Alkaline phosphatase was prepared from Escherichia coli (strain CW 3747) according to the method described by Applebury et al. (25). Enzyme concentrations were determined spectrophotometrically at 278 nm with εM280 = 0.72 (29). Molar calculations are based on a molecular weight of 86,000 (28). Apoalkaline phosphatase was prepared by Chelex treatment of the native enzyme as previously described (18). All equipment and solutions were prepared metal-free according to published procedures (19). Pipes’ (monosodium salt, monohydrate) buffer was obtained from Calbiochem (La Jolla, Calif.). Metal ion solutions were prepared from spectrographically pure chloride salts purchased from Johnson, Matthey, Ltd., London, England. All other chemicals employed were reagent grade. Apophosphoryl alkaline phosphatase was prepared by phosphorylation of the Cd2+ enzyme and subsequent removal of metal ion as previously described (28).

Calorimetry — Calorimetric measurements were made on a Privalov scanning adiabatic calorimeter at a heating rate of 1° min−1 (30). Detailed procedures for scanning calorimetry follow those previously described (1, 4). For most experiments samples were heated from 20 to 80°C. To effect transitions of the metalloalkaline phosphatase samples were heated to 100°C. Following heating, samples were cooled to 20°C which required a time period of 0.5 h. Except where indicated, reheating of samples was initiated immediately on equilibration at 20°C. The calorimetric scans displayed record the differential heat capacity of samples as a function of temperature. Prior to loading of the sample chambers, the system was flushed thoroughly with metal-free buffers to remove trace contaminants of divalent metal ion.

Circular dichroism spectra were taken on a Cary 60 recording spectropolarimeter with a 6001 circular dichroism attachment using a 5-mm jacketed cell.

RESULTS

Apoenzyme — Calorimetric scans of solutions of apoalkaline phosphatase at pH 7.5 are shown in Fig. 1A. Over a 20-fold range of protein concentration (0.31 to 6.57 mg ml−1) a single transition is observed with a transition temperature (Tm) of 57.5°C. The differential heat change, ΔCp, is a linear function of concentration (Fig. 2) corresponding to a specific enthalpy, ΔH, of 5.57 cal g−1 (Table I). Following cooling to 20°C, the samples were immediately rescanned under the same conditions to yield the results shown in Fig. 1B. In all but the most dilute samples two transitions are clearly resolved, occurring at transition temperatures of 57.5°C (identical to that observed on initial heating) and 43.5°C. Repetitive scans of the reheated sample duplicate the heat capacity profile observed on second heating with respect to the position and relative amplitude of the transitions. The initial heating of the protein thus appears to convert the sample into two species undergoing thermally induced transitions at characteristic temperatures. The species undergoing the 43.5°C transition is metastable. If the interval between successive thermal scans is increased from 0.5 to 9 h, the amplitude of the 43.5°C transition is reduced, while the 57.5°C transition has increased and approaches that observed on initial heating (Fig. 3A; Table I). Under these conditions induction of the thermal transitions does not result in irreversible denaturation. On cooling to ambient temperature no precipitation is observed, and the optical spectrum (CD) of the protein and recovery of catalytic activity on Zn2+ addition are unchanged. It is therefore reasonable to identify the 57.5°C transition observed on reheating with apoprotein which has returned to its native state. Given the value of the differential heat capacity (ΔC) and differential heat change (ΔQ), Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5. ○, apoalkaline phosphatase, 57.5°C transition; ●, apoalkaline phosphatase, 44.0°C transition; □, apophosphoryl alkaline phosphatase, 65.0°C transition; ●, apophosphoryl alkaline phosphatase, 44.0°C transition; ■, apophosphoryl alkaline phosphatase, 60.0°C transition.

specific enthalpy for this transition, ΔH = 5.57 cal g−1, and assuming that no denaturation has occurred, the concentration of protein associated with the 43.5°C transition can be calculated (Table I). The differential heat change of this transition is a linear function of protein concentration (Fig. 2) corresponding to a specific enthalpy, ΔH = 2.01 cal g−1.

Except for the most dilute samples, the calculated distribution of protein between the native and metastable forms appears to be independent of concentration. On immediate reheating 70 ± 3% of the protein is in the intermediate form.
Immediate reheating

h). B), apophosphoryl alkaline phosphatase: l), initial heating; 2), immediately reheating.

Equilibration of the species undergoing the observed transition (cf. Table III). A), initial heating; 2), immediately reheating.

Concentration dependence of differential heat change ($\Delta Q_h$) for thermal transitions of apoenzyme (E) and apophosphoryl alkaline phosphatase (E-P).

The conditions were: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; samples were heated from 20-80°C for first and second heatings.

| First heating | Second heating |
|---------------|----------------|
| $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ | $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ | $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ |
| mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ | mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ | mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ |
| 0.31 | 1.83 | 0.63 | 0.31 |
| 0.59 | 3.38 | 1.26 | 0.59 |
| 1.09 | 6.63 | 1.74 | 0.79 |
| 2.19 | 13.27 | 3.98 | 1.50 |
| 3.57 | 22.12 | 5.65 | 4.90 |
| 6.57 | 42.88 | 9.65 | 1.67 |
| 7.76 | 78.99 | 13.99 | 2.51 |

* Second heating initiated 0.5 h after completion of first heating.

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Fig. 3. Time dependence of thermally induced transitions. Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; Numerals I to V refer to the species undergoing the observed transition (cf. Table III). A), apoenzyme than the observed transition; 1), initial heating; 2), immediate reheating.

Table I

Concentration dependence of differential heat change ($\Delta Q_h$) for thermal transitions of apoenzyme (E) and apophosphoryl alkaline phosphatase (E-P).

The conditions were: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; samples were heated from 20-80°C for first and second heatings.

| First heating | Second heating |
|---------------|----------------|
| $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ | $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ | $|E|P|$ | $\Delta Q_{45.2'}$ | $\Delta Q_{55.2}$ |
| mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ | mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ | mg ml⁻¹ | kcal mol⁻¹ | kcal mol⁻¹ |
| 0.31 | 1.83 | 0.63 | 0.31 |
| 0.59 | 3.38 | 1.26 | 0.59 |
| 1.09 | 6.63 | 1.74 | 0.79 |
| 2.19 | 13.27 | 3.98 | 1.50 |
| 3.57 | 22.12 | 5.65 | 4.90 |
| 6.57 | 42.88 | 9.65 | 1.67 |
| 7.76 | 78.99 | 13.99 | 2.51 |

* Second heating initiated 0.5 h after completion of first heating.

Differential Scanning Calorimetry of Alkaline Phosphatase

Fig. 4. Thermally induced transitions of apophosphoryl alkaline phosphatase (Apo AP-P); effect of variation of protein concentration. Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; protein concentrations as indicated. A), initial heating; B), immediate reheating.

and 30 ± 3% has returned to the native state. This proportion is constant over almost a 10-fold concentration range. The absence of a concentration dependence suggests that the conversion of the intermediate to the native form can be described as a first order process occurring with a half-time of ~7 h ($k_{100\text{KDa}} = 4 \times 10^{-5} \text{ s}^{-1}$). At the very lowest concentrations the apparent deviation (Fig. 1B) from this behavior may result from a reduction in the rate of conversion of protein into the native state (i.e. the 57.5°C transition) on cooling. The reason for this slower conversion of the refolded apoprotein from the metastable state to the native state is unclear.

Apophosphoryl Enzyme—Calorimetric scans of the apophosphoryl enzyme in which the serine of one of the subunits has been specifically phosphorylated are given in Fig. 4. A single transition occurring at a temperature higher than that of the apoenzyme is observed on initial heating. As in the case of the apoenzyme the transition temperature ($T_1 = 58^\circ$) and specific enthalpy ($\Delta h = 4.20 \text{ cal g}^{-1}$) show no dependence on protein concentration (Figs. 2 and 4A; Table I). On immediate reheating two transitions are observed (Fig. 4B1). In contrast to the behavior of the apoenzyme, however, neither transition temperature ($44^\circ$ and 60°) corresponds to that observed initially. The low temperature transition can be identified with a metastable intermediate which slowly converts to the form undergoing the 60° transition. As with the apoenzyme, following a prolonged interval between successive scans, the amplitude of the low temperature transition is decreased while that of the high temperature transition is increased (Fig. 4B1; Table I). The initial 65° transition, and by inference the native state of the apophosphoryl enzyme, does not reappear even on prolonged equilibration. The failure of the apophosphoryl enzyme to return to the native state is consistent with the reduction in the catalytic activity which can be restored to the enzyme on addition of Zn⁺.

In contrast to the apoenzyme, only ~50% of the specific activity is restored to the apophosphoryl enzyme on the addition of Zn⁺ following heating.

Since neither of the transitions detected on reheating correspond to the transition observed in the initial thermal scan, analysis of the system is not straightforward. If it is assumed that no irreversible denaturation has occurred, the data can be fit by assigning values of specific enthalpy, $\Delta h$, of 1.90 cal g⁻¹ to the 44.0° transition and $\Delta h = 4.00 \text{ cal g}^{-1}$ to the 60.0° transition.
transition (Table I). In contrast to the behavior of the apoenzyme, the time-dependent generation of the species undergoing the high temperature transition is markedly concentration-dependent (Table I). The experimental variations preclude any attempt to determine the apparent order of the conversion process based on the available data.

Effects of Metal Ion Binding — The effect of metal ion addition on the calorimetric behavior of the apoenzyme is given in Fig. 5. Addition of 1 eq of Zn\(^{2+}\) does not alter the observed transition temperatures on initial or second heating but reduces the amplitude by ~50%, corresponding to a halving of the concentration of protein capable of undergoing thermally induced processes (Table II). Two equivalents of Zn\(^{2+}\) are sufficient to completely eliminate the appearance of transitions in the first heating. On reheating, however, a transition at 44.0° is observed, equivalent to about 55% of the total protein. The presence of 4 eq of Zn\(^{2+}\) abolishes all transitions associated with the apoenzyme on both first and second heatings. The effect of the second 2 eq of Zn\(^{2+}\) on the protein structure is also reflected in a marked reduction in the heat capacity over the temperature range of the calorimetric scan.

Addition of 1 eq of Cd\(^{2+}\) to the apoenzyme yields results similar to those observed on Zn\(^{2+}\) addition (Table II; Fig. 6);

![Fig. 5. Effect of Zn\(^{2+}\) concentration on thermally induced transitions of apoalkaline phosphatase (Apo AP). Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; protein concentration, 3.76 mg ml\(^{-1}\) (4.51 \times 10\(^{-4}\) M). Zn\(^{2+}\) was added to the samples as Zn\(^{2+}\)Cl\(_2\), to yield the indicated [Zn\(^{2+}\)]/[apoalkaline phosphatase] ratios. A), initial heating; B), immediate reheating.](image)

![Fig. 6. Effect of metal ion addition on thermally induced transitions of apoalkaline phosphatase. Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; protein concentration, 3.78 mg ml\(^{-1}\) (4.51 \times 10\(^{-4}\) M), metal ions were added as Me\(^{2+}\)Cl\(_2\). The transition observed on initial heating is displayed. A), apoalkaline phosphatase (Apo AP); B), apoalkaline phosphatase + Zn\(^{2+}\), [Zn\(^{2+}\)]/alcaline phosphatase = 0.99; C), apoalkaline phosphatase + Cd\(^{2+}\), [Cd\(^{2+}\)]/apoalkaline phosphatase = 1.15; D), apoalkaline phosphatase + Mg\(^{2+}\), [Mg\(^{2+}\)]/apoalkaline phosphatase = 1.18.](image)

**Table II**

| Effect of metal ion addition on differential heat change \(\Delta Q_{T_m}\) for thermal transitions of apoalkaline phosphatase |
|---------------------------------------------------------------|
| The conditions were: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; samples were heated from 20–80° for first and second heatings. |
|                      | First heating | Second heating |
|----------------------|---------------|----------------|
|                      | \(\Delta Q_{T_m}\) | \(\Delta Q_{T_m}\) |
|                      | mol cal g\(^{-1}\) | mg cal g\(^{-1}\) | mol cal g\(^{-1}\) | mg cal g\(^{-1}\) |
| Zn\(^{2+}\)          | 21.56         | 10.73          | 7.17             | 4.96             |
| Zn\(^{2+}\)          | 19.80         | 0.00           | 0.00             | 0.00             |
| Zn\(^{2+}\)          | 3.95          | 0.00           | 0.00             | 0.00             |
| Cd\(^{2+}\)          | 1.15          | 15.36          | 0.00             | 0.00             |
| Mg\(^{2+}\)          | 1.18          | 24.10          | 0.00             | 0.00             |

* Second heating was initiated 0.5 h after completion of first heating.
* Enzyme concentration ([E]) = 3.87 mg ml\(^{-1}\).
* Me\(^{2+}\) was added to samples as solutions of Me\(^{2+}\)Cl\(_2\).
* \([E]_e = \text{calculated enzyme concentration derived from values of}\)  

\[\Delta Q_{T_m}, \text{i.e. } [E]_e = \Delta Q_{T_m}/\Delta h_{T_m}, \text{where } \Delta h_{T_m} \text{ refers to values obtained for apoalkaline phosphatase in the absence of metal ion (cf. Table I).} \]

\[\Delta Q_{T_m} = [E]_{13\to15} - [E]_{13\to15} \text{ at } T_m = 61.3, \text{ } \\
\Delta Q_{T_m} = [E]_{13\to15} - [E]_{13\to15} \text{ at } T_m = 69.0\]
temperature, $T_m$, corresponds to the denaturation change in heat capacity, $\Delta C_p^m$, which can be independently assessed from the calorimetric trace at a given pH (7). While a rigorous assessment of $\Delta C_p^m$ derived from the transitions at various pH values was not performed, the value of $\delta \Delta H/\delta T_m = \Delta C_p^m$ obtained from the limited data of this study is 0.19 cal deg$^{-1}$ g$^{-1}$, in good agreement with the value of $\Delta C_p^m$ determined from the calorimetric scans (Table III).

High Temperature Transitions of Metalloenzymes — The presence of $\geq$2 eq of Zn$^{2+}$ is sufficient to abolish the transitions observed with the apoenzyme (Fig. 5). Extension of the thermal scans to extreme temperature ($\sim$100$^\circ$) permits detection of the transitions of the metallophosphatases. The metal ion stoichiometry has a pronounced effect on the nature of the observed transitions as reflected in both the $T_m$ and transition enthalpy (Fig. 8; Table III).

In the presence of 2 eq of Zn$^{2+}$, a broad, slightly asymmetric transition is observed, $T_m = 89.1^\circ$. The presence of a second 2 eq of Zn$^{2+}$ results in a transition at $T_m = 88.8^\circ$. Addition of 1 eq of Mg$^{2+}$ raises the transition temperature to 90.0$^\circ$. As the transition temperature increases, the enthalpy associated with the transition also increases to roughly twice that of the apoenzyme (Table III).

At a protein concentration of $\sim$2.7 mg/ml an asymmetric transition with an apparent $T_m$ of 96$^\circ$ is observed for the native enzyme (Table III). Dilution of this sample by a factor of 2 permits resolution of the overall transition into two apparently symmetric components with $T_m$ values of 89.8 and 93.0$^\circ$, respectively. The specific enthalpy of the native protein is markedly greater than that of the regenerated metalloenzymes, suggesting that some component contributing to the stabilization of the structure of the native enzyme is absent in the reconstituted protein.

Thermally Induced Transitions of Apo- and Metalloalkaline Phosphatases as Detected by Circular Dichroism — The thermally induced transitions are also reflected in changes in the circular dichroism spectrum of the protein. As the temperature of the apoenzyme is increased from 20 to 80$^\circ$, the value of $\theta_{222}$ is reduced, showing a linear relation with temperature below 30$^\circ$ and above 70$^\circ$ (Fig. 9). Between 30 and 70$^\circ$ there is an increase in the reduction of $\theta_{222}$, which appears to follow a sigmoidal curve with a midpoint at 58$^\circ$, corresponding to the value of $T_m$ observed in the calorimetric scans. While the spectroscopic transition is ultimately reversed completely, there is a hysteresis in the recovery of ellipticity as the

![Fig. 7. pH dependence of transition temperature observed on initial heating of apopalkaline phosphatase. Conditions: O, 0.01 M sodium citrate, 0.01 M HPO$_4$, 0.1 M NaCl; 0, 0.01 M Tris, 0.01 M sodium acetate, 0.1 M NaCl; O, 0.01 M Pipes, 0.1 M NaCl; protein concentration, 2 to 4 mg ml$^{-1}$; final pH 7.4.](http://www.jbc.org/)

| Table III: Calorimetric and thermodynamic parameters of apo-, apophosphoryl, and metalloalkaline phosphatases.

| Species                  | Initial state | $T_m$ ($^\circ$C) | $\Delta H$ (cal g$^{-1}$) | $\Delta C_p$ (cal deg$^{-1}$ g$^{-1}$) | $\Delta H_{\text{m}}$ (kcal mol$^{-1}$) | $n$ | $\Delta S$ (cal deg$^{-1}$ g$^{-1}$) | $\Delta H_{\text{eff}}$ (kcal mol$^{-1}$) | $\Delta S_{\text{eff}}$ (cal deg$^{-1}$ g$^{-1}$) |
|-------------------------|---------------|-------------------|---------------------------|-------------------------------------|--------------------------------------|-----|------------------------------------|--------------------------------------------|------------------------------------------|
| I                       | Apoalkaline phosphatase | 57.5 | 5.57 | 0.196 | 0.53 | 4.42 | 0.18 | -0.02 | 0.23 | 13.56 |
| II                      | Apoalkaline phosphatase | 43.5 | 2.01 | 0.177 | 1.02 | 1.97 | 0.38 | -0.14 | 0.03 | 2.33 |
| III                     | Apophosphoryl alkaline phosphatase | 65.0 | 4.20 | 0.196 | 0.54 | 4.22 | 0.26 | -0.90 | 0.06 | 5.59 |
| IV                      | Apophosphoryl alkaline phosphatase | 44.0 | 1.90 | 0.177 | 0.98 | 2.63 | 0.58 | -0.20 | 0.03 | 2.40 |
| V                       | Apophosphoryl alkaline phosphatase | 60.0 | 4.00 | 0.074 | 0.67 | 2.98 | 1.78 | 0.50 | 0.26 | 22.22 |
| VI                      | Zn$^{2+}$ alkaline phosphatase | 86.0 | 7.17 | 0.225 | 0.45 | 6.49 | 5.80 | 1.08 | 1.07 | 89.99 |
| VII                     | Zn$^{2+}$ alkaline phosphatase | 86.0 | 7.17 | 0.225 | 0.45 | 6.49 | 5.80 | 1.08 | 1.07 | 89.99 |
| VIII                    | Mg$^{2+}$ alkaline phosphatase | 90.0 | 9.91 | -    | 0.62 | 3.87 | 4.81 | 2.38 | 1.51 | 129.46 |

$^a$ Parameters derived from the observed transitions, the specific transition enthalpy ($\Delta H_n$), the change in heat capacity on denaturation ($\Delta C_p$), the transition temperature ($T_m$), and the peak height ($\Delta H_m$) were determined as previously described (7).

$^b$ $\Delta H_{\text{m}}/\Delta H_n$, the ratio of the van't Hoff enthalpy change ($\Delta H_n$) to the calorimetric enthalpy change ($\Delta H_m$) was determined from the transition parameters as previously described (7).

$^c$ $\Delta H_{\text{m}}/\Delta T_m = \Delta C_p$, where $R$ is the gas constant, $\Delta C_p$ is the transition amplitude at $T_m$, and $M_r$ is 86,000 g mol$^{-1}$. For these calculations $T_d$ is assumed to be equal to $T_m$.

$^d$ $\Delta H_n$ (kcal mol$^{-1}$) = $\Delta H_{\text{m}}$ (cal g$^{-1}$) g mol$^{-1}$ (10$^{-3}$ kcal cal$^{-1}$).

$^e$ For determination of thermodynamic parameters, a value of $M_r = 0.085$ cal deg$^{-1}$ g$^{-1}$ was used.

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FIG. 8. Effects of metal ion composition on thermally induced transitions of alkaline phosphatase. Conditions: 0.01 M Pipes, 0.1 M NaCl, pH 7.5; protein concentration, 2.10 mg ml⁻¹ (2.44 × 10⁻⁴ M). The transitions observed on initial heating are displayed. Metal ion compositions of the samples whose approximate stoichiometry is indicated on the figure are: apo-alkaline phosphatase (Apo AP) (no metal ions); apo-alkaline phosphatase + 2 Zn²⁺ ([Zn⁺²/sapoprotein] = 2.18); apo-alkaline phosphatase + 4 Zn⁺² ([Zn⁺²/sapoprotein] = 4.36); apo-alkaline phosphatase + 4 Zn⁺² + 1 Mg²⁺ ([Zn⁺²/Mg⁺²]/[apoalkaline phosphatase] = 4.36, [Mg⁺²]/[apoalkaline phosphatase] = 1.33). Numerals I and VI to VIII refer to species undergoing the observed transitions (cf. Table III).

The sample is cooled. Paralleling the calorimetric result, the nonlinear transition is abolished on addition of 2 eq of Zn⁺² to the sample. These results are qualitatively similar to analogous experiments reported for the temperature-dependent circular dichroism spectra of pancreatic ribonuclease (31).

DISCUSSION

Cooperativity of Transitions Mechanism of Reversible Unfolding—While the mechanism of protein unfolding and folding remains a highly controversial subject, the denaturation of small globular proteins, as analyzed on the basis of thermodynamic criteria, can be approximated by a two-state model (7, 32). That is the transition from native to denatured state has been observed to occur with no population of intermediate states of significant thermodynamic stability. Appro-

Deviation from the two-state model has been documented for the thermally induced unfolding of ribonuclease A at pH > 4 (4). Under the conditions of these experiments, however, unfolding is not completely reversible, which may account for the apparent decrease in cooperativity (4). The refolding of chemically (guanidinium hydrochloride) denatured carbonic anhydrase B has also been reported to deviate from the two-state model (33, 34). However, the relationship of these results to thermal denaturation is unclear.

In contrast, the reversible transitions of apo- and apophosphoryl alkaline phosphatase deviate markedly from this model. The value of ΔHₚ/ΔHₗ for the transition of native alkaline phosphatase (I, Fig. 3A) observed on initial heating is 0.53 corresponding to n = 4.42 (Table III). The two-state model precludes the formation of intermediates of significant thermodynamic stability in either the unfolding or folding process; thus the appearance of a metastable intermediate on reheating of the sample is consistent with n > 2 for the unfolding of I. While ΔHₚ/ΔHₗ for the high temperature transition is unaltered on reheating, ΔHₚ/ΔHₗ associated with the low temperature transition is 1.02 (n = 1.97) in excellent agreement with the two-state model.

For apophosphoryl alkaline phosphatase, the induced transition of the native form (III, Fig. 3B) of the protein yields a value of ΔHₚ/ΔHₗ of 0.54 (n = 4.22), similar to that observed for the apoenzyme and again deviating from the predicted behavior of the two-state model. As for the apoenzyme, an
intermediate (IV, Fig. 3B) undergoing a low temperature transition is observed on reheating whose unfolding corresponds to the two-state model \( \Delta H_{m}/\Delta H_{c} = 0.98, n = 2.03 \). Unlike the apoenzyme, however, intermediate IV does not return to III, the native state, but is slowly converted to an additional intermediate, V, whose transition temperature is below that of III (the native state) (Fig. 3). For the unfolding of V, \( \Delta H_{m}/\Delta H_{c} = 0.67, n = 2.98 \). Thus, there appears to be a monotonic decrease in the number of states associated with the unfolding of III, V, and IV, suggesting a sequential conversion of these species in denaturation.

These data suggest that, despite marked differences in the properties of the apo- and apophosphoryl enzymes, the gross mechanism of reversible unfolding is similar and can be described as indicated in Fig. 10. For both proteins, initial conversion of N, the native state, to U, the unfolded state present at high temperature is a four-state transition. The absence of marked deviations in calculated estimates of the total protein present in various forms (Table I) suggests that on cooling all protein is converted to a state other than U. The presence of a state, identified with N", which undergoes a two-state transition to U for both proteins indicates that k_2 must be sufficiently small (the kinetic energy barrier must be sufficiently large) to permit trapping of the protein in this state. N" is thermodynamically metastable and converts to a different state with a half-time on the order of hours (Fig. 3; Table I).

For the apoenzyme N" is apparently converted very rapidly to N since a transition corresponding to N" is not detectable. For the apophosphoryl enzyme, N" is converted into a state identified with N' which undergoes a three-state transition (Table III). Since the appearance of N is not detected for the apophosphoryl enzyme even on prolonged equilibration at low temperature, k_1 <<< k_2, k_3 for the modified protein. Trapping of the refolded apophosphoryl enzyme as V, the N' state, could arise either as a result of the kinetics of refolding or represent a thermodynamic state of enhanced stability (see below). In either case it is clear that, while the gross mechanism of unfolding and refolding of the unmodified and phosphorylated apoproteins is similar, the presence of a single phosphoryl group on the dimer substantially alters the relative stability of intermediates and, by implication, their structure at the molecular level.

**Thermodynamic Parameters and Structural Alterations of Intermediates**–Thermodynamic parameters can be determined from the calorimetric data (Table III). Privalov has demonstrated that the magnitude of the change in heat capacity \( \Delta C_p \) is proportional to the extent to which nonpolar contacts stabilize the structure (7). For small globular proteins the normalized values of \( \Delta C_p^*/C^* \) range from 0.09 to 0.16 cal deg^-1 g^-1. The transitions accompanying unfolding of both the apo and apophosphoryl alkaline phosphatases show significantly greater values of \( \Delta C_p^*/C^* \approx 0.20 \) cal deg^-1 g^-1, suggesting a high degree of hydrophobic interactions in these structures. The value of \( \Delta C_p^*/C^* \) is also anomalously high for the transitions of intermediates II and IV, but is reduced to a more normal value for the transition of the apparently stable species V (\( \Delta C_p^*/C^* = 0.07 \) cal deg^-1 g^-1).

The proposed mechanism of unfolding and refolding of the apoproteins (Fig. 10) is in accord with the calculated values of the free energy difference at 30°, separating the folded (N,N',N") and unfolded (U) states of the species (Table III). The apoenzyme is stabilized relative to the denatured state by \(-20 \) kcal mol^-1, while the postulated metastable intermediate, II, is much less stable with a free energy lying \(-2.5 \) kcal mol^-1 below the denatured state. Thus the transitory formation of II leading to ultimate renaturation of I appears consistent with the thermodynamic states of these species.

While the mechanism of unfolding of the native state (N) of the apo- and apophosphoryl proteins appears similar, the consequences of phosphorylation on the thermodynamic stability of corresponding states is dramatic. The native state of the apophosphoryl enzyme is not a markedly stable species, lying but \(-5.6 \) kcal mol^-1 below the denatured state. Thus, assuming that the denatured states of both proteins are of roughly equivalent energy, the apophosphoryl enzyme is destabilized relative to the apoprotein by \(>14 \) kcal mol^-1. Metastable intermediate IV appears to be analogous to the corresponding species II in the refolding of the apoprotein with a free energy just below that of the denatured state. Intermediate V, which does not convert to the native state III, is remarkably stable lying \(-22 \) kcal mol^-1 below the denatured state and \(-14.4 \) kcal mol^-1 below the native state of the apophosphoryl enzyme. Thus V is a thermodynamic well in the refolding process.

**Fig. 10.** Free energy levels of conformational states of alkaline phosphatases. N, N', N", and U refer to states identified in the postulated mechanism of unfolding (see text). For description of states I to VIII see Table III. Starred (*) levels are those of the apophosphoryl enzyme states.
anism of refolding of the apophosphoryl enzyme is such that
the polypeptide chain forms a structure of relatively high
internal order (V) which is sufficiently stable to prevent
significant reformation of the native state on the time scale of
these experiments.

State of Subunit Aggregation in Intermediates – Calcula-
tions of the calorimetric enthalpies assume that the species
undergoing transitions is the dimer (Mₐ = 86.000). Since the
parameters (ΔH, ΔC_p) describing the calorimetric transitions
are independent of protein concentration, it is unlikely that
oligomeric forms of protein other than the dimer are involved.
Equilibrium sedimentation studies of the native and apopro-
teins at concentrations >1 mg ml⁻¹ show a single symmetrical
peak corresponding to the dimer molecular weight (17, 20).
Despite evidence suggesting that the native state of the
apo-protein is a dimer with extensive subunit interactions, it
is not implausible that the protomers might undergo ther-
mainly induced unfolding as apparently independent species.
The evidence supporting the existence of negative homotro-
mi interactions in the enzyme, however, indicates that the post-
tulated conformational identity of the subunits is abolished
on phosphorylation at the active site of one of the two subunits
in the dimer. Thus, two structurally distinct classes of subunit
are present in the apophosphoryl enzyme. The transitions of
both the apo- and apophosphoryl proteins are markedly simi-
lar (compare degrees of cooperativity and values of ΔC_p).
Table III) despite the difference in the structure of the associ-
ated subunits. If dissociated monomers were undergoing sepa-
rate transitions, resolution of transitions arising from the
two classes of subunit of the apophosphoryl protein might be
anticipated. However, the transitions of the apophosphoryl
enzyme are symmetrical despite the difference in the nature
of associated subunits. It appears reasonable, therefore, to
consider the dimer as the fundamental unit undergoing tran-
sitions.

On the other hand, if the monomer is taken to be the fun-
damental unit, deviation of ΔH_m/ΔH_u from unity is re-
duced, but not abolished. For the transition of state I of the
apoenzyme ΔH_m/ΔH_u is 0.74, assuming a molecular weight of
43.000. Thus, the presence of intermediate states is still
required. However, the value of ΔH_m/ΔH_u for the transi-
tions of the metastable intermediates II and IV is 1.45 when
calculated on the basis of the monomer molecular weight,
indicating that the molecular weight of the cooperative unit
of this species is necessarily greater than that of the isolated
subunit. Since these intermediates (II and IV) represent the
final state of the protein prior to complete unfolding, this
suggests that all transitions represent structural alterations
of dimeric states.

While the dimer appears to be the species undergoing the
observed transitions, the nature of the state of association in
the final unfolded state (U) cannot be assessed directly. Given
the apparently rapid restoration of dimeric aggregation to the
sample on cooling, identification of U as either protein mono-
mers or weakly associated dimers appears equally possible.
However, the renaturation of the apophosphoryl enzyme,
resulting in the formation of species which are not equivalent
energy of an intermediate species can result in a reduction in the
kinetic barriers separating intermediates on the reaction pathway.
Thus, if structural alterations in the corresponding states of
the apoproteins, the conformational changes induced on ligand
binding and phosphorylation (negative cooperativity) might prevent
the phosphoserine from adopting a form of sufficient thermodynamic
stability to seriously limit the rate of the overall reaction.

to the species formed on refolding of the apoenzyme indicates
that either thermally induced unfolding does not result in
complete dissociation (i.e. physical separation) of subunits, or
that the process of refolding and reassociation occurs with a
remarkable fidelity to the native state.

Full dissociation of the apophosphoryl enzyme would result
in generation of unassociated subunits (unphosphorylated
monomers) capable of reassociating to form the apoenzyme
dimer. The transition of apoenzyme (I) is not, however,
observed on reheating samples of the apophosphoryl protein.
Thus, subunit dissociation would require that unfolded, un-
modified subunits derived from the phosphoryl enzyme differ
from unfolded, unmodified subunits derived from the nonphos-
phorylated apoenzyme. While the fidelity of subunit associa-
tion in heterogeneous mixtures of unfolded polypeptides has
been documented (e.g. aldolase) (38-40), retention, on refold-
ing, of the conformation induced on modification at a neigh-
boring subunit would be extraordinary. Perhaps as remarka-
ble is the alternative explanation, that physical separation of
discrete subunits does not occur at a temperature of 80°. This
would require that the subunit-subunit association domain is
the structural element of greatest stability in the protein.
Since heating to 100° results in irreversible changes in the
structure of the apo- and metalloenzymes (in contrast to the
reversibility of processes induced at the lower temperature),
it appears that failure to totally dissociate the dimer at lower
temperatures is the more likely explanation.

Enzyme Structure and Unfolding Reaction – Available
structural information on alkaline phosphatase provides some
rationale for the unusual behavior of the apoproteins. The
amino acid composition of the enzyme shows a moderate
number of nonpolar residues and a relatively high proline
content (40 residues/dimer) (9). Two intradimer disulfide
bridges are critical to maintenance of the structural integrity
of the protein. These cysteine bridges can be reversibly re-
duced (41), but oxidation or alkylation of the cysteine group
results in irreversible formation of disordered monomer at
room temperature (42). It is therefore possible the structural
changes in protein structure which occur only at temperatures
>80° are associated with modification of the cysteine residues.

Analysis of the CD spectra of the native and aprotein
suggests that the secondary structure contains only a small
proportion of α helix and a large contribution (~60%) of β
structure (17). Analytical assessment by the Chou and Fas-
man method (43, 44) of the elements of secondary structure
derived from the amino acid sequence currently available4
are in rough agreement with the estimate of α helix based on

4The criterion of irreversibility in experiments in which samples
are heated to 100° is the failure of the sample to display transitions,
on reheating, whose transition enthalpy corresponds to the protein
content of the sample. By this standard, heating of apo- and
metalloalkaline phosphatases to 100° is irreversible even after equil-
ibration at 20° for >10 h. In all cases, however, transitions of low
enthalpy are observable, occurring at the transition temperature of
the native protein. Since heating to ~100° alters the reversibility of
renaturation and hence the state of the denatured protein, it is
possible that reassembly of molecular structure occurs (i.e. the
process is reversible), but at an extremely slow rate. The rate of
reassembly of oligomeric enzymes is highly variable (39, 40).
Deter-
mination of the nature of changes effected in the unfolded state by
further thermal denaturation must await future experimental inves-
tigation.

5Analysis of the structure is based on the sequence as presently
available from the work of R. A. Bradshaw, P. A. Neuman, F.
Cancedda, K. Schrili, J. D. Hecht, and M. J. Schlesinger cor-
responding to ~90% of the primary structure of the enzyme (personal
communication from R. A. Bradshaw).
spectroscopic methods, but do not predict extended regions of β structure. Since the analytical methods have been criticized for overestimating the length of regions of β structure (45), it is striking that numerous small regions of β structure scattered throughout the sequence are predicted by such an analysis. The predicted absence of large structural domains may be related to the high number of proline residues in the molecule (46) which are known to destabilize both α helical and β structures (43). While the energy of isomerization of proline residues is not sufficient to account for the stabilization of refolded intermediates (35), it is possible that, in a structure comprised of a large number of ordered structural domains, proline isomerization could be coupled to conformational changes involving a number of amino acid residues. Amplification of the energetic requirements for such a transition to permit kinetic and thermodynamic stabilization of intermediates is plausible in a highly segmented but ordered structure.

The temperature-dependent changes in the circular dichroism spectrum of the apoenzyme reflect a large but not complete loss of ordered structure (Fig. 9). As in the corresponding calorimetry experiment (Fig. 5), the spectroscopic transition is abolished by the addition of 2 eq of Zn'+/enzyme dimer to the system. Retention of structural elements on thermal denaturation, in contrast to the total unfolding of the polypeptide inducible by guanidinium chloride, has been documented for other proteins (47). Acid denaturation of alkaline phosphatase, occurring on exposure of the protein to an environment of pH < 4.0 differs from thermal unfolding, yielding completely dissociated subunits which are completely unfolded (17). In agreement with these observations, no thermal transitions are detected in the calorimetric scans of solutions of protein at pH < 4.0. The hysteresis accompanying recovery of structure on reversal of the exposure of the protein to low pH has also been confirmed in these studies. This hysteresis has been attributed to alterations in the reversibility of the titration of carboxyl groups which become exposed in the unwound, dissociated structure resulting in an apparent shift in the pKₐ of these groups (17). Deprotonation, apparently required for refolding to the native state, becomes possible only at high pH and the consequent alteration of the surface charge is suggested as responsible for structural alterations preventing full recovery of quaternary structure, i.e. the reassociation of monomers is complete only at pH > 6.0.

The observation of a hysteresis in the reversal of the thermal denaturation as monitored by circular dichroism might be construed as evidence that the mechanism of refolding of thermally and acid-denatured protein are related. The marked differences in the nature of the denatured states (i.e., degree of apparent association of subunits and the extent to which structure is retained) suggests that such a comparison, in the absence of more detailed information, is unwarranted.

**Effects of Metal Ion Binding on Heat-induced Transitions — Addition of Zn'**

Addition of Zn' to solutions of the apoenzyme results in a reduction of the amount of apoprotein undergoing the thermally induced transitions (Figs. 5 and 8; Table II). The decrease in the observed thermal transition enthalpy is proportional to the amount of metal ion added and is complete at a Zn'+ concentration corresponding to the presence of 2 eq of Zn'+/enzyme dimer. A variety of experimental methods have demonstrated that population of specific metal ion binding sites of high affinity and unusual structure is complete at a metal ion equivalence of 2/apoprotein dimer (15, 16). Similarly 2 eq of metal ion/apoprotein dimer are necessary and sufficient to restore full functional capacity as measured by the stoichiometry of ligand (e.g., phosphate, aryl phosphonate) binding to the enzyme (27). These results suggest that metal ion binding is a cooperative process, i.e., that formation of the two metal ion protein selectively occurs as opposed to random population of high affinity binding sites. The calorimetric titration is consistent only with the preferential formation of the two Zn'+ enzymes. This is true even in the absence of ligand and the presence of excess apoprotein (Fig. 5). Thus, association of metal ion at the active center binding sites must be a positively cooperative process. The same conclusion appears to apply to formation of the Cd'+ enzyme (Fig. 5). In contrast, the stabilization of the enzyme afforded by Mg'+ requires the initial presence of the Zn'+ ions (Figs. 5 and 8).

The specific enthalpies of all of the metallophosphatases (VI to VIII, Fig. 8; Table III) are substantially greater than that of the apoenzyme, while the values of ΔC° are markedly reduced (0.02 to 0.04 cal·deg⁻¹·g⁻¹). The calculated thermodynamic parameters (Table III) indicate that metal ion binding stabilizes the folded protein relative to the denatured state by 90 to 130 kcal mol⁻¹ in contrast to the modest (20 kcal mol⁻¹) stabilization of the protein in the absence of metal ion. The value of the entropy change, ΔS, between the states is also increased for the metalloenzymes, indicating a substantial increase in the degree of ordered structure relative to the apoprotein. The decrease in the value of ΔC° is consistent with a reduction of nonpolar interactions on metal ion binding. The dramatic changes in the thermostability and thermodynamic properties are primarily a consequence of the binding of the first 2 eq of Zn'+ to the apoprotein (Fig. 8). Subsequent metal ion additions produce additional stabilization (Table III).

The binding of metal ions to apoa alkaline phosphatase has been shown to result in alterations in the conformational stability of the enzyme reflected in changes in solvent (H₂O) access to structural elements of the protein. The binding of 2 or 4 eq of Zn'+ increases from 120 to 200 the number of protons which are not in rapid exchange with solvent as measured by hydrogen-tritium exchange (22). A number of these protons are susceptible to exchange occurring at a slow rate, leaving an inert "core" of protons which are virtually completely shielded from solvent. The number of shielded protons present in the apoenzyme is not increased on binding 2 eq of Zn'+. However, the presence of 4 eq of Zn'+ increases the number of exchange-inert protons by a factor of 3 (22). Bioskynthesized incorporation of m-fluorotyrosine and [γ-¹⁴C]histidine into alkaline phosphatase has permitted assay by ¹⁹F and ¹³C NMR methods (48, 49) of the effect of metal ion binding on the environment of individual amino acid residues. Well resolved resonances are observed in spectra of the native enzyme dimer corresponding to the number of tyrosine or histidine residues per monomer. Thus, corresponding residues on separate subunits appear to be symmetry-related. The magnitude of the chemical shifts reflect the local environment of the amino acids. Removal of the metal ions results in changes in the spectra consistent with a reduction in ordered structure and an increased susceptibility of the protein structure to solvent penetration. Restoration of the well resolved pattern of resonances is largely complete on addition of 2 eq of Zn'+ to the apoprotein, but the presence of 4 eq of Zn'+ appears necessary to duplicate precisely the spectrum of the native enzyme.

The interpretation of the calorimetric data in thermodynamic terms is consistent with these observations. There appears to be a collapse of ordered elements of structure on
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removal of the metal ion. To compensate for the loss of defined structure, the apoproteins adopt conformations which increase the association of hydrophobic groups as reflected in the increase in heat capacity (ΔC_p) (Table III) (7). There is a concomitant exposure of polar groups to enhanced solvent interaction as indicated by the NMR data (48, 49). Nevertheless, the removal of the metal ions results in an enormous decrease in thermodynamic stability.

The multistate mechanism for the unfolding of alkaline phosphatase is maintained on metal ion binding. In fact, the addition of the first 2 eq of metal ion decreases the cooperativity of the observed transition (ΔH_m/ΔH_e (VI) = 0.45). Subsequent metal ion additions increase the cooperativity moderately (ΔH_m/ΔH_e (VII) = 0.53, ΔH_m/ΔH_e (VIII) = 0.62), but in no case does the system correspond to the two-state model. This suggests that as structural constraints are imposed on the enzyme, as a consequence of sequential population of metal ion binding sites, the energetic separation of thermodynamically significant states is decreased.

These data indicate that occupation of distinct metal-ion binding sites results in formation of chemically distinct species (VI to VIII). Binding of Zn^{2+} at the tight binding sites associated with the two active centers of the dimer is sufficient to restore functional capacity and structural integrity to the protein and is necessary before the consequences of metal ion association at other binding sites can become effective (Fig. 8). Both structure and function depend primarily on occupancy of the active center binding sites (50), but both structural and catalytic efficiency appear to be positively affected by occupancy of the additional binding sites for Zn^{2+} and Mg^{2+} (12, 51, 52).

Experiments conducted on samples of native enzyme gave results qualitatively similar to those observed for the reconstituted metalloalkaline phosphatases. However, the value for the transition enthalpy was significantly higher (ΔH = 16.0 cal g⁻¹) and multiple transitions, occurring at high temperatures (T_m > 90°), were resolved as an apparent function of decreasing enzyme concentration. As isolated, alkaline phosphatase is known to be heterogenous with respect to metal ion and phosphate content. Given the effect of metal ion stoichiometry on the transition temperature and transition enthalpy it is reasonable to attribute the detected differences in the native and reconstituted protein to the heterogeneity of the enzyme as isolated.

The relationship of these studies to the nature of the folding and subunit association of alkaline phosphatase occurring in vivo is difficult to assess. The biosynthetic generation is a complex process. The site of enzyme synthesis appears to be the form of the protein isolated from the periplasmic space (53). The detailed pattern of structural changes preceding and accompanying generation of the biosynthetic product remains to be determined.

Acknowledgments We wish to thank Dr. Joseph E. Coleman and Dr. Julian M. Sturtevant for support, suggestions, and discussion.

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