

- Ogawa, Y., Quagliarotti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., & Busch, H. (1969) *J. Biol. Chem.* 244, 4387-4392.
- Paik, W. K., & Kim, S. (1980) *Protein Methylation*, Wiley, New York.
- Plapp, B., Rafferty, M., & Cole, R. D. (1967) *J. Biol. Chem.* 242, 265-270.
- Roth, M. (1971) *Anal. Chem.* 43, 880-882.
- Sanborn, B. M., & Hein, G. E. (1968) *Biochemistry* 7, 3616-3623.
- Sasagawa, T., Okuyama, T., & Teller, D. C. (1982) *J. Chromatogr.* 240, 329-340.
- Suh, B.-S., Lee, H. W., Hong, S.-Y., Kim, S., & Paik, W. K. (1986) *Anal. Biochem.* (in press).
- Van Holde, K. E., & Isenberg, I. (1975) *Acc. Chem. Res.* 8, 327-335.

## Studies of Thermally Induced Denaturation of Azurin and Azurin Derivatives by Differential Scanning Calorimetry: Evidence for Copper Selectivity<sup>†</sup>

Helen R. Engeseth and David R. McMillin\*

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907

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**ABSTRACT:** Azurin, a blue copper protein from *Pseudomonas aeruginosa*, and several derivatives of azurin have been studied by differential scanning calorimetry. Two well-separated, irreversible transitions are observed in a scan of apoazurin under a variety of conditions, and they are assigned to distinct steps in the denaturation process. No specific structural component can be assigned to the lower temperature transition, but a "flap" structure which is found near the metal binding site may be involved. Circular dichroic spectra suggest that melting of the  $\beta$ -sheet structure, the main structural motif in the native protein, occurs during the second transition. With the exceptions of the Ni(II) and *p*-(hydroxymercuri)benzoate derivatives, the transitions are superposed in the metalated forms, and the enthalpies of denaturation are more endothermic. By comparison with other first-row divalent transition ions and especially Zn(II), the Cu(II) derivative exhibits the most endothermic denaturation process. Along with other data, this suggests that the binding energy is greater for Cu(II). It is postulated that the selectivity for copper over zinc arises because of the irregular binding geometry offered by the folded protein. Denaturation of the Hg(II) derivative is even more endothermic, confirming that the type 1 binding site has a very great affinity for Hg(II). Finally, when substoichiometric amounts of Hg(II) are added to the apoprotein, there is evidence that a novel mercury-bridged dimer of azurin forms.

The type 1 or "blue" copper center occurs in a number of proteins and has quite distinctive spectroscopic properties. Its hallmark is an intense visible absorbance in the region of 600 nm where the molar absorptivity is typically ca. 5000 M<sup>-1</sup> cm<sup>-1</sup>. A second characteristic signature is an unusually small hyperfine splitting from the copper nucleus in the so-called parallel region of the electron paramagnetic resonance spectrum (Fee, 1975; Gray & Solomon, 1981). The small blue copper proteins like plastocyanin, which is found in chloroplasts, and azurin, which is a bacterial protein, are believed to function as electron transferases (Farver & Pecht, 1984; Adman, 1985). These proteins tend to occur at the high-potential end of electron-transport systems; accordingly, the reduction potential of a blue copper center is fairly positive and usually greater than that of the aqueous Cu(II)/Cu(I) couple (Gray & Solomon, 1981).

The spectroscopic properties can be rationalized in terms of structure, now that crystal and molecular structures are available for the oxidized forms of plastocyanin from *Populus nigra* var. *italica* (Gus & Freeman, 1983) and the azurins from *Pseudomonas aeruginosa* (Adman & Jensen, 1981) and *Alcaligenes denitrificans* (Norris et al., 1983). The plastocyanin structure is known in most detail, and it reveals that there are

four ligands disposed in a distorted tetrahedral fashion about copper. The donor set is comprised of two nitrogens from imidazole groups and a cysteine sulfur, each 2.0-2.1 Å removed from Cu(II), and a methionine sulfur which is found significantly farther away (ca. 2.9 Å). In the azurins, the copper environment is similar except that a fifth donor, a peptide oxygen, may be close enough to interact with the copper. The presence of the cysteine sulfur gives rise to a low-energy sulfur to copper charge-transfer absorption which is primarily responsible for the intense visible absorbance (Gray & Solomon, 1981; McMillin et al., 1974; McMillin & Morris, 1981). The tetrahedral coordination geometry along with covalent interactions involving the thiolate ligand explains the narrow hyperfine splitting (Brill, 1977; Malmström & Vänngård, 1960; Bencini & Gatteschi, 1983; Penfield et al., 1985).

The protein structure also influences the redox reactivity of the copper center. The prevailing view is that by presenting a rather rigid, distorted tetrahedral binding site, the protein conformation provides for a relatively positive reduction potential and a small Franck-Condon barrier for the electron-transfer reaction (Gray & Solomon, 1981; Farver & Pecht, 1981, 1984; Holwerda et al., 1976; Gray & Malmström, 1983). Because Cu(II) is generally found in a tetragonal environment with a coordination number of 5 or 6, the blue copper site appears to be biased toward the binding of Cu(I). In addition,

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analysis of the d-d absorption bands of the oxidized proteins reveals that the ligand-field stabilization energy is not optimal and suggests that the strain energy is about 70 kJ mol<sup>-1</sup> (Gray & Solomon, 1981; Gray & Malmström, 1983). Comparing reduction potentials of the proteins and model compounds gives a somewhat smaller strain estimate, ca. 30 kJ mol<sup>-1</sup> (Addison et al., 1984).

The question of the importance of strain at the Cu(II) center and its effect on the electron-transfer process is only one aspect of the larger problem of understanding the functional significance of structure in blue copper proteins (McMillin & Engeseth, 1985). For example, we have pointed out that the blue copper site binds Cu(II) more readily than it does other (divalent) first-row transition-metal ions (Blaszak et al., 1983). The selectivity must have a structural basis but could be a kinetic or a thermodynamic effect. Of course, the existence of strain is counterproductive from the point of view of selectivity since it decreases the affinity for Cu(II). A comparison of the binding constants for Co(II), Cu(II), etc. would reveal how significant the strain is in relation to other factors. Unfortunately, the binding constants are difficult to obtain for kinetic reasons (Blaszak et al., 1983). In an attempt to obtain some insight into relative metal ion binding energies, we have undertaken studies of the thermally induced denaturation of azurin and various metal-substituted derivatives of azurin by the method of differential scanning calorimetry. Previous studies of metalloenzymes have shown that the presence of the metal ion stabilizes the folded form of the protein and hence has a marked influence on denaturation (Coleman & Chlebowski, 1979; Chlebowski & Mabrey, 1977; Donovan, 1984). Our results show that thermally induced denaturation of azurin is not a simple two-state process and that the process is irreversible. This precludes the measurement of the free energy of metal binding. Nevertheless, the calorimetric studies provide some information about relative binding energies.

#### EXPERIMENTAL PROCEDURES

**Materials.** The bacterial culture of *Pseudomonas aeruginosa* was obtained from the American Type Culture Collection (strain 10145). Fresh spinach leaves (*Spinacea*) were used as the source of plastocyanin. The acetone powder of the latex from the Chinese natural lacquer tree (*Rhus vernicifera*) was purchased from Saito and Co., Ltd., Osaka, Japan. Atomic absorption standards, purchased from American Scientific Products, were used as sources for the metal ions. 5,5'-Dithiobis(2-nitrobenzoic acid) (dtnb)<sup>1</sup> was purchased from Aldrich Chemical Co., the sodium salt of *p*-(hydroxymercuri)benzoic acid (pmb) was purchased from Sigma Chemical Co., and both were used without further purification. All buffers were prepared from reagent-grade chemicals and distilled, deionized water and were passed through a Chelex-100 (Bio-Rad) column prior to use.

**Methods.** The bacteria were cultivated, and native azurin was isolated as previously described (Ambler & Brown, 1967). Protein purity was checked by ultraviolet spectroscopy and SDS gel electrophoresis as well as by monitoring the absorption ratio between the bands at 626 and 280 nm. The ratio was typically 0.4–0.5. Plastocyanin (Markley et al., 1975) and stellacyanin (Reinhammar, 1970) were extracted and purified according to published methods. Apoplastocyanin was pre-

pared as before (McMillin et al., 1974). The copper was removed from azurin with thiourea (Blaszak et al., 1983). The metal derivatives of azurin were prepared by the addition of a slight excess of metal ion to the apoprotein in 50 mM ammonium acetate, pH 9.0. After incubation for 24 h, the protein was dialyzed into 20 mM sodium phosphate, pH 8.0. In the titration experiments, portions of the metal ion were added directly to the apoprotein solution in 20 mM sodium phosphate, pH 8.0. The thiol derivatives were prepared by the addition of the appropriate thiol-specific reagent to the apoprotein according to literature procedures (Boyer, 1954; Ellman, 1959). This was followed by dialysis into 20 mM sodium phosphate, pH 8.0. The pH titrations of the native proteins and apoproteins were accomplished in a buffer 20 mM each in borate, citrate, and phosphate. All dialyses were carried out in hollow fiber devices. During dialysis, the temperature was controlled between 5 and 10 °C by a thermoelectric stirrer. Protein concentrations were determined either by the procedure of Lowry (Lowry et al., 1951) (bovine serum albumin from Sigma Chemical Co. was used as a standard) or from the absorbance at 280 nm using the molar absorptivity from the literature (Tennent & McMillin, 1979).

The enthalpy of denaturation was computed from the area under the transition recorded by the calorimeter (Privalov, 1982). The area was determined by the cut and weigh method, and the weight was converted to an enthalpy with the use of a previously determined calibration factor. The heating rate in these experiments was 1 °C/min.

**Instrumentation.** Electronic absorption spectra were recorded on a Cary 17D spectrophotometer. The pH was measured at room temperature with a Radiometer/Copenhagen PHM 64 pH meter. Heat capacity measurements were obtained on a Microcal-1 differential scanning calorimeter (Amherst, MA) equipped with matched 1-mL platinum cells. Circular dichroism measurements were conducted on a Cary 61 spectrophotometer in a cell of 1-cm path length.

#### RESULTS

**Apoazurin.** In all studies of the apoprotein over a wide range of protein concentrations (0.2–3.6 mg mL<sup>-1</sup>), two separate melting phenomena have been observed (Figure 1A). The  $T_m$  values of both transitions are pH dependent below pH 6, where  $T_m$  is the temperature at which the heat capacity exhibits a local maximum. However, in the range of pH 6–9, the  $T_m$  of the second transition remains constant (Figure 2). Ultraviolet CD spectra are presented for three samples of apoazurin in Figure 3. All three spectra were run at room temperature, but the samples had different thermal histories. The CD spectrum of unheated apoazurin exhibits a minimum at 221 nm in line with previous work (Tang et al., 1969). For the sample which has been heated to 75 °C, the minimum is shallower and is shifted to shorter wavelength. The sample which has been heated to 100 °C exhibits a still shallower minimum at an even shorter wavelength. After 5 min at 75 °C, apoazurin shows a pronounced decrease in its ability to recombine with Cu(II); only about 35% of the visible absorbance returns. If a sample of apoazurin is rescanned in the calorimeter, neither transition is observed in the second trace.

Figure 4 shows how modification of the thiol group influences the calorimetric profile of apoazurin. If dtnb is bound to the protein, both transitions shift to lower temperature. If pmb is bound, the first transition is not strongly perturbed, but the second shifts to lower temperature. Azurin contains three cysteines, but two are involved in the disulfide 3–26 bridge (Adman & Jensen, 1981). Hence, the thiol labels are assumed to bind at Cys-112, one of the ligands in the copper

<sup>1</sup> Abbreviations: dtnb, 5,5'-dithiobis(2-nitrobenzoic acid); M<sup>II</sup>Az, metalated form of azurin; pmb, *p*-(hydroxymercuri)benzoic acid; SDS, sodium dodecyl sulfate.

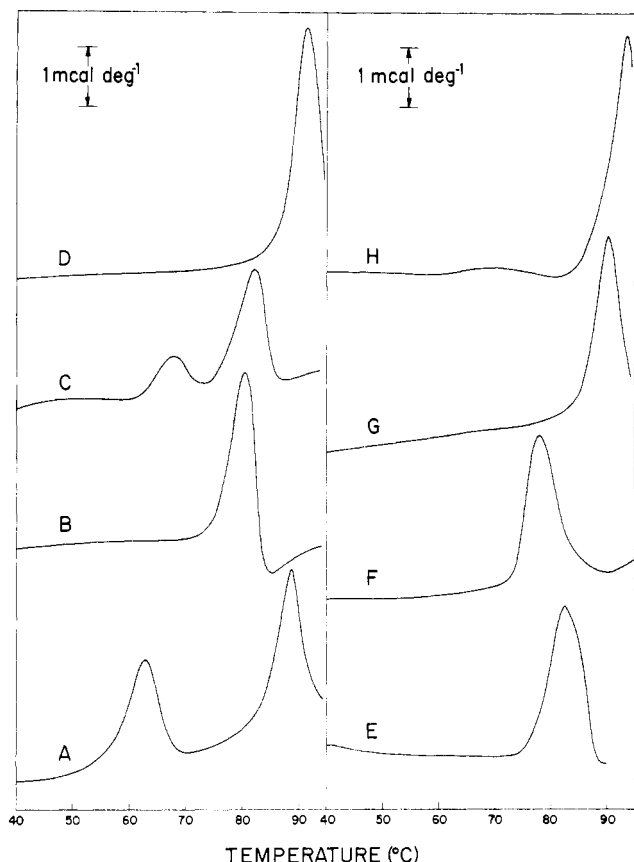


FIGURE 1: Calorimetric scans of azurin and azurin derivatives in 0.02 M pH 8 phosphate. (A)  $3.06 \text{ mg mL}^{-1}$  apoazurin; (B)  $0.75 \text{ mg mL}^{-1}$   $\text{Cu}^{\text{II}}$ Az; (C)  $1.48 \text{ mg mL}^{-1}$   $\text{Ni}^{\text{II}}$ Az; (D)  $1.00 \text{ mg mL}^{-1}$   $\text{Hg}^{\text{II}}$ Az; (E)  $0.93 \text{ mg mL}^{-1}$   $\text{Co}^{\text{II}}$ Az; (F)  $1.28 \text{ mg mL}^{-1}$   $\text{Ag}^{\text{I}}$ Az; (G)  $1.40 \text{ mg mL}^{-1}$   $\text{Zn}^{\text{II}}$ Az; (H)  $0.86 \text{ mg mL}^{-1}$   $\text{Cd}^{\text{II}}$ Az.

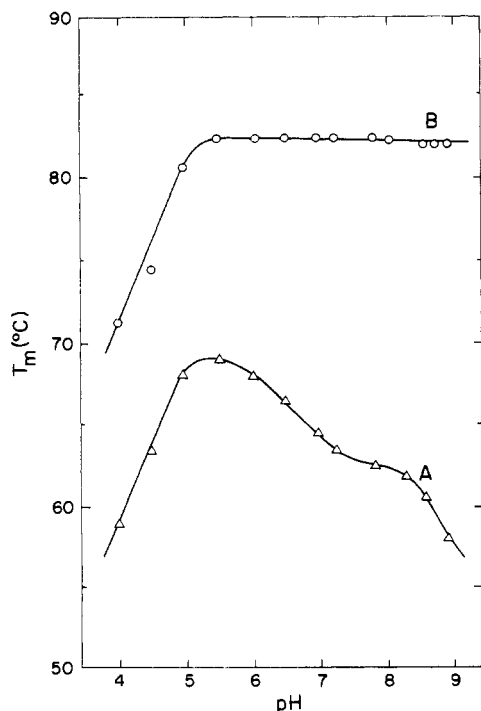


FIGURE 2: pH dependence of maxima in calorimetric scans of apoazurin. (A) First transition; (B) second transition.

binding site. Consistent with this assumption, previous work has shown that when pmb is bound to apoazurin, the characteristic blue color of native azurin does not develop on the addition of  $\text{Cu}(\text{II})$  (Tennent & McMillin, 1979).

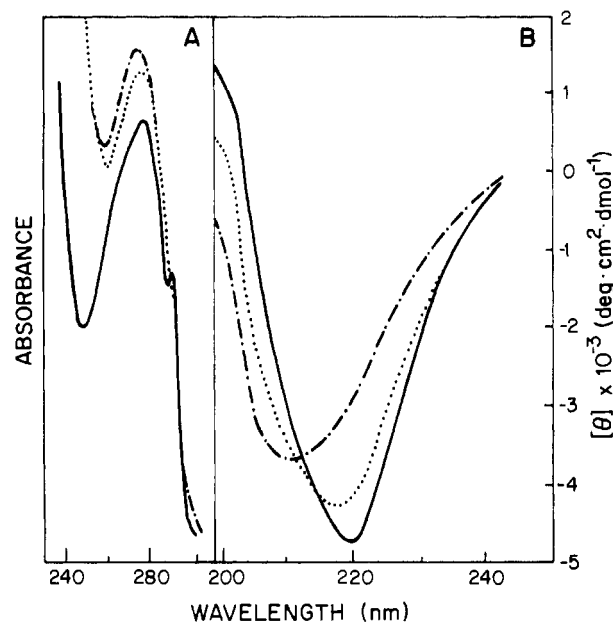


FIGURE 3: Absorbance and CD spectra of apoazurin samples as a function of thermal history. All spectra were run at  $25^\circ\text{C}$  in 0.02 M pH 8 phosphate. (—) Sample that was unheated; (···) sample that had been heated to  $75^\circ\text{C}$ ; (---) sample that had been heated to  $100^\circ\text{C}$ .

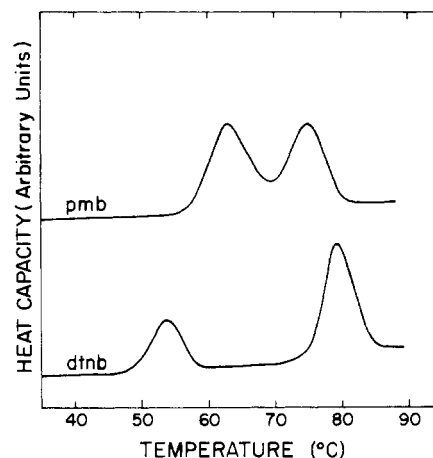


FIGURE 4: Calorimetric scans of apoazurin samples which have been treated with sulfhydryl reagents. The buffer is 0.02 M pH 8 phosphate.

**Apoplastocyanin and Apostellacyanin.** As controls, the denaturation of apoplastocyanin and apostellacyanin was also examined by differential scanning calorimetry. Apostellacyanin—like apoazurin—exhibited two transitions, with  $T_m$  values of  $59$  and  $90^\circ\text{C}$ , respectively. For plastocyanin, a single, rather broad transition occurred in the range of  $65$ – $70^\circ\text{C}$ .

**Metal Ion Derivatives of Azurin.** Figure 5 shows how the two transitions of apoazurin disappear in parallel as increasing amounts of  $\text{Hg}(\text{II})$  are added to the sample. In the initial stages of the titration, a new transition appears at ca.  $83^\circ\text{C}$ . As more mercury is added, this transition begins to be replaced by another, higher temperature transition. Finally, when a stoichiometric amount of  $\text{Hg}(\text{II})$  is present, a single transition occurs at  $91^\circ\text{C}$ . If  $\text{Cu}(\text{II})$  is titrated into apoazurin in an analogous fashion, the interpretation is less straightforward due to the overlap among transitions. Nevertheless, the results suggest that an intermediate form also occurs when sub-stoichiometric levels of  $\text{Cu}(\text{II})$  are added.

Denaturation is clearly a more cooperative process when a full equivalent of  $\text{Cu}(\text{II})$  or  $\text{Hg}(\text{II})$  is bound to the protein since

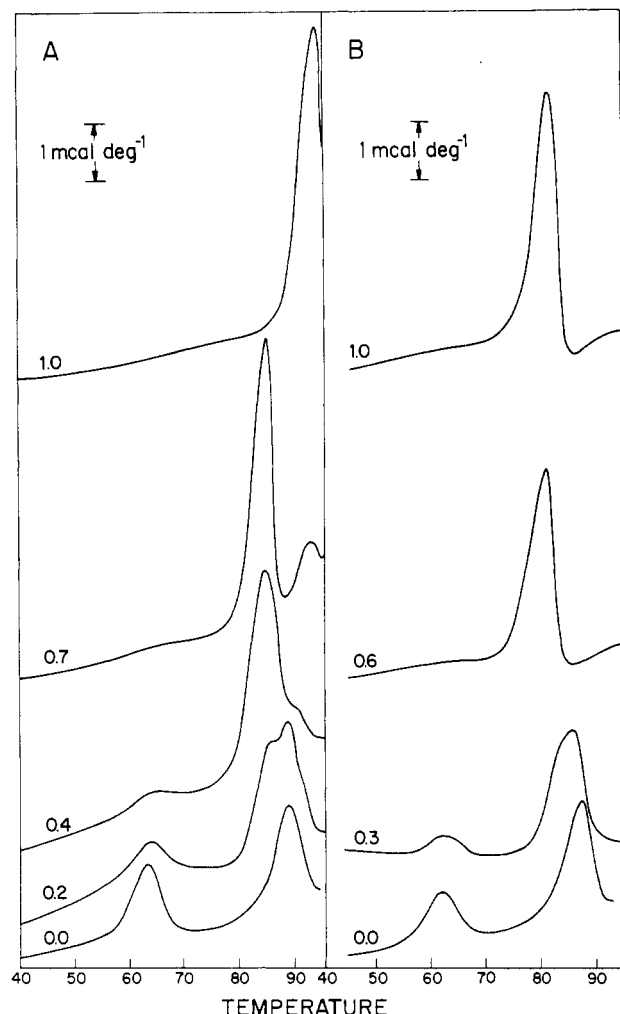


FIGURE 5: Calorimetric scans of azurin samples as a function of the ratio of metal ion to protein in 0.02 M pH 8 phosphate. (A) Hg(II) added; (B) Cu(II) added.

Table I: Calorimetric Data for Azurin and Azurin Derivatives<sup>a</sup>

sample	$\Delta H_d$ (cal g <sup>-1</sup> )	$T_m$ (°C)
apoazurin	4 (1)	62 (2) <sup>b</sup>
	6 (1)	86 (2) <sup>b</sup>
Cu <sup>II</sup> Az	>21	80 (2)
Co <sup>II</sup> Az	21 (2)	82 (2)
Ni <sup>II</sup> Az <sup>c</sup>	6 (1)	75 (2)
	10 (1)	90 (2)
Ni <sup>II</sup> Az <sup>d</sup>	3 (1)	68 (2)
	7 (1)	82 (2)
Zn <sup>II</sup> Az	12 (2)	90 (2)
Ag <sup>I</sup> Az	13 (2)	78 (2)
Hg <sup>II</sup> Az	23 (2)	91 (2)
Cd <sup>II</sup> Az	24 (3)	93 (2)

<sup>a</sup>Uncertainties in the estimated values are given in parentheses.

<sup>b</sup>Average of nine trials. <sup>c</sup>Contains 1 equiv of excess Ni(II). <sup>d</sup>No excess Ni(II) added.

two transitions are no longer resolved. The results in Figure 1 graphically demonstrate that a variety of other metal ions also bind to apoazurin and similarly influence the denaturation process. The  $T_m$  values and the relevant thermodynamic data are compiled in Table I. Except for the Co(II) and Ni(II) derivatives, the metalated forms tend to exhibit a single transition. In the case of the Co(II) derivative, the calorimetric profile exhibits a single maximum, but there is a pronounced shoulder on the high-temperature side. The scan of the Ni(II) derivative most nearly resembles that of apoazurin in that two well-separated transitions occur, but the  $T_m$  values fall between

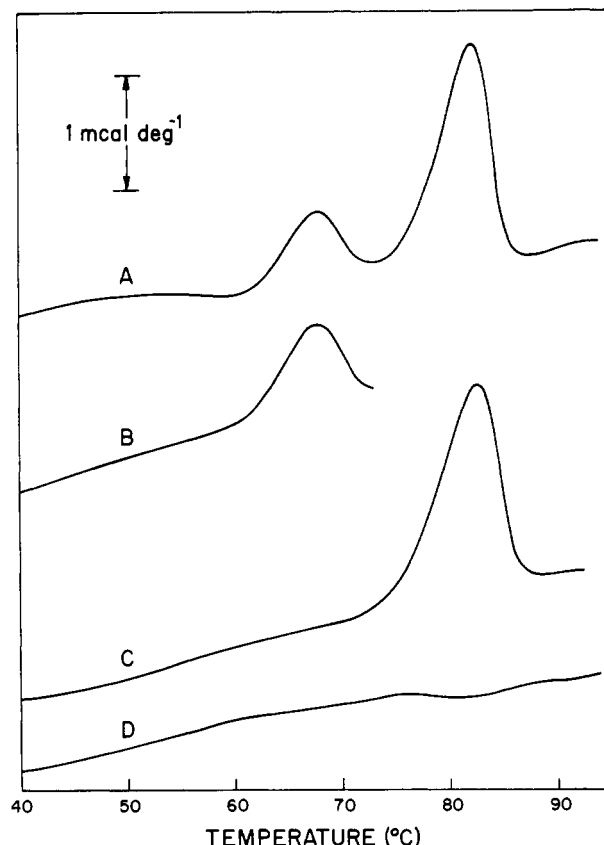


FIGURE 6: Calorimetric scans of Ni<sup>II</sup>Az samples in 0.02 M pH 8 phosphate. (A) Normal scan; (B) scan stopped at 73 °C; (C) rescan of (B) after cooling; (D) rescan of (A) after cooling.

those of apoazurin. Sequential heating experiments based on the nickel(II) derivative (Ni<sup>II</sup>Az) show that the transitions are separate, irreversible processes (Figure 6). Thus, after the protein has been heated to 75 °C (through the first transition) and cooled overnight, the second transition is the only one observed upon rescanning. In a subsequent scan, neither transition appears.

## DISCUSSION

**Calorimetry of Apoazurin.** In principle, the transitions in the calorimetric profile of apoazurin could arise from two different forms of apoazurin in solution, or they could reflect a two-stage denaturation of the folded protein. Several observations argue against the former interpretation. In the first place, the two transitions of apoazurin are lost in parallel as Hg(II) combines with the protein (Figure 5). If two forms of apoazurin were present, this result would require that, fortuitously, both species have the same affinity for Hg(II). Moreover, the first transition represents only about 40% of the total heat of denaturation, but we found that 65% of the protein failed to reconstitute after a solution of apoazurin had been heated to 75 °C. Finally, Ni<sup>II</sup>Az exhibits analogous transitions, and the sulfur to nickel charge-transfer absorbance from *all* protein in solution is lost upon heating through the first transition.

The other possibility, multistage denaturation, can be described by the minimum reaction scheme:



and



where N denotes the folded state which is capable of binding

copper to give native azurin, D is the completely denatured state which is formed on heating through both transitions, and I is an intermediate state which is formed as a result of the first transition. Multistage denaturation has been observed in a number of proteins including pepsin (Privalov et al., 1981) and cytochrome P-450 (Anzenbacher et al., 1982), but these systems usually contain distinct domains that fold in a quasi-independent fashion (Privalov et al., 1981).

Before discussing possible structures for species I and D, it is necessary to consider the structure of apoazurin. Although no direct structural information is available,  $^1\text{H}$  NMR results suggest that the apoprotein is folded very much like native azurin (Hill & Smith, 1979), and X-ray studies have shown that the Cu(II) protein is a compact globular structure built from a single polypeptide chain (Adman & Jensen, 1981; Adman et al., 1978). Most of the residues combine to form a  $\beta$  sandwich which is flared at the end where copper is located (Adman et al., 1978). One of the  $\beta$  sheets contains three strands, which are composed mainly of residues from the C-terminal end of the chain; the other sheet is composed of five strands. The only other well-defined unit of structure is a largely helical segment of about 28 residues which is extruded from the sandwich and which functions as a flap covering a hydrophobic area on one layer of the  $\beta$  sandwich, near the metal binding site. Thus, there are no spatially isolated domains in the azurin molecule.

In principle, CD spectra can provide some information about the species I and D. In general  $\beta$ -sheet structure is characterized by a single negative trough around 220 nm whereas an  $\alpha$  helix exhibits two minima (Sakena & Wetlaufer, 1971). Figure 3 reveals that there is extensive  $\beta$ -sheet structure, and relatively little  $\alpha$  helix, in both species N and I, as is the case in native azurin. It seems likely that the  $\beta$  structures in species N and I involve many of the same residues. The reason is that the original  $\beta$ -sandwich (or  $\beta$ -barrel) structure is widely distributed and is believed to be a quite stable folded unit (Richardson, 1984).

The structure in the neighborhood of the metal binding site is apparently altered as a result of eq 1 because protein which has been heated to 75 °C shows a sharply decreased ability to bind Cu(II) and Ni(II). The pH studies shown in Figure 2 also suggest that the structure at the metal binding site is affected by eq 1. Over the pH range of 6–9, where histidine side chains are titrated, the  $T_m$  value of the first denaturation transition varies while that of the second remains constant. This is significant because three of the protein's four histidine residues are intimately associated with the metal binding site—His-46 and His-117 are ligands, and His-35 is probably hydrogen bonded to one of the copper ligands (Canters et al., 1984). Below pH 6, both  $T_m$  values decrease with decreasing pH. This may be due to the protonation of carboxyl-containing side chains, groups which are distributed fairly evenly about the surface of the protein (Freeman, 1981).

Since the  $\beta$  structure appears to survive the first stage of denaturation, it is tempting to ascribe this step to the flap moiety, the only other easily recognized structural unit in native azurin. The fact that apoplastocyanin, which lacks the flap structure, exhibits but one transition is consistent with this assignment. Even if the flap structure is involved in the first stage of denaturation, some modification of the  $\beta$  sandwich probably also occurs because the flap covers a hydrophobic surface on one of the  $\beta$  sheets.

Parenthetically, it can be noted that the calorimetry data for the various apoproteins suggest that the structure of stellacyanin may be much more like that of azurin than

plastocyanin. Previous  $^{113}\text{Cd}$  NMR studies have pointed to the same conclusion insofar as the active site is concerned (Engeseth et al., 1984).

**Irreversibility.** It is interesting that the denaturation of a small protein like azurin is irreversible when many other larger, seemingly more complex proteins exhibit reversible denaturation (Privalov, 1982). A straightforward explanation is available if the unfolding process—which itself might be reversible—is coupled to another highly irreversible process, for example, some type of protein aggregation process. In particular, as azurin contains a cysteine sulfhydryl group, the unfolded protein may be susceptible to dimerization via disulfide formation. It may be significant in this regard that the denaturation of native azurin is unique in that it exhibits a trailing exothermic process (Figure 1). Compared with the other metals investigated, copper is expected to be the best catalyst for the oxidation of thiol groups (Takagi & Isemura, 1964).

A comparison of the measured enthalpy of denaturation ( $\Delta H_d$ ) and the van't Hoff enthalpy of denaturation ( $\Delta H_{vH}$ ) gives an indication of the number of molecules which are involved in the cooperative process (Donovan, 1984). The  $\Delta H_{vH}$  is easy to calculate, if a reversible, two-state equilibrium can be assumed (Privalov, 1982). Although the irreversible nature of the denaturation of azurin makes the argument less convincing, the calculated  $\Delta H_d/\Delta H_{vH}$  ratios are typically 2–2.5 and are consistent with some type of dimerization process.

**Calorimetry of Metalated Forms of Azurin.** For most of the derivatives, there is good evidence that the metal ion binds at the type 1 site. To summarize some of the salient evidence, an analysis of the charge-transfer absorption spectra of  $\text{Co}^{\text{II}}\text{Az}$  and  $\text{Ni}^{\text{II}}\text{Az}$  reveals that the metal is attached to the cysteine thiolate group (Tennent & McMillin, 1979). Moreover,  $^1\text{H}$  NMR and Raman studies indicate that the histidine ligands are present as well (Hill & Smith, 1976; Blaszk et al., 1982; Musci et al., 1985; Ferris et al., 1984). In the case of  $\text{Hg}^{\text{II}}\text{Az}$ , we have found that Hg(II) blocks copper uptake and that is masks the thiolate group which can be titrated in apoazurin. In addition, Hg(II) has been shown to displace copper and to lodge in the copper binding site of the related protein, plastocyanin (Colman et al., 1978). There is good evidence that cadmium is found at the copper binding site from  $^{113}\text{Cd}$  NMR spectroscopy, thiol titrations, and competitive binding studies (Engeseth et al., 1984). Finally, the fact that the various metals have similar effects on the calorimetric profile of the protein argues that the metals all compete for a common binding site.

When the binding is weak, as it is in  $\text{Ni}^{\text{II}}\text{Az}$ , vide infra, two distinct transitions are observed, but the transitions are superposed in most of the derivatives. Separate transitions are also observed in the thiol-labeled derivatives as seen in Figure 4. These results suggest that strong binding and the formation of bonds to multiple ligands of the binding site are required to superpose the transitions. Because the denaturation is not a reversible process, the  $T_m$  values are not readily related to thermodynamic state functions. Consequently, we will only attempt to interpret the enthalpy data in Table I. Even these values must be approached with caution, since there is no guarantee that completely parallel processes occur for the various metal derivatives. In the simplest case, denaturation involves a transition from a folded to an unfolded state with complete dissociation of the metal ion. Whether or not the metal ion dissociates from the denatured protein in each case is not known. Indeed, as can be seen from the  $\Delta H_d$  values in Table I, the measured enthalpy of denaturation of  $\text{Ni}^{\text{II}}\text{Az}$  is

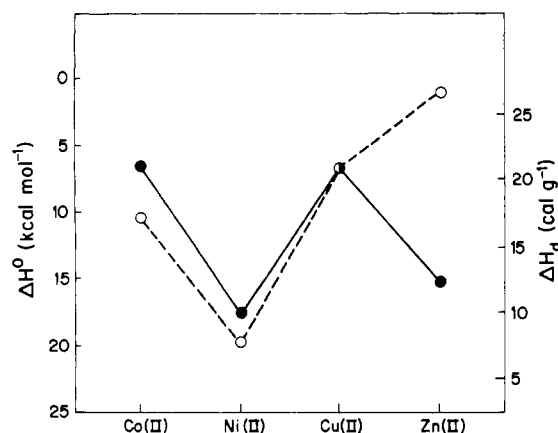


FIGURE 7: Enthalpies of formation of tetrabromide complexes in water as a function of metal ion at 25 °C (O). Enthalpies of denaturation of azurin derivatives at the respective melting temperatures (●). The two ordinate scales were arbitrarily placed to force the Cu(II) data points to coincide.

apparently increased when excess Ni(II) is present. Moreover, if protein aggregation occurs in the denatured state, *vide supra*, the degree of association could vary from case to case.

In spite of these uncertainties, the enthalpy data appear to give an indication of the relative stabilities of the various metalated forms. In particular, the enthalpy data suggest that Hg(II) stabilizes the folded form of the protein more than Cu(II) and that either of these derivatives is more stable than the Ni(II) derivative (Table I). Other, indirect observations support this ordering. The fact that mercury displaces copper from plastocyanin (Colman et al., 1978) strongly suggests that the type 1 site has a higher affinity for Hg(II) than Cu(II). While entropy considerations may play a role, in view of the affinity Hg(II) has for thiolate ligands, it seems likely that the enthalpy term is also important. As regards Ni(II), <sup>1</sup>H NMR studies reveal that this metal—unlike Cu(II) or Hg(II)—readily dissociates from the protein at lower pH values (Blaszak et al., 1982). Furthermore, when a significant concentration of strongly complexing buffer like imidazole is present, Ni(II) is not taken up by apoazurin (Blaszak et al., 1983). These results indicate that the formation constants of the respective azurin derivatives follow the order Ni(II) < Cu(II) < Hg(II), the same order which is predicted by the calorimetry data.

If we take the calorimetrically determined enthalpies to be indicative of relative binding energies, the enthalpies of the first-row transition ion derivatives, along with Zn<sup>II</sup>Az, make an interesting comparison. The inherent trend in stabilities of tetrahedral complexes of these ions can be gauged from the results of Bianchi and Paoletti (1985), who have studied the hydrolysis of the corresponding tetrabromide adducts (Figure 7). They inferred that entropy effects were essentially constant throughout the series and that the enthalpy data strongly correlated with the free energies. The formation of the tetrabromide adduct is most favored in the case of Zn(II) and least favored in the case of Ni(II). The relative instability of the nickel complex is readily understood in terms of simple ligand-field theory which shows that the loss in ligand-field stabilization energy as a result of the change from octahedral to tetrahedral symmetry is greatest in the case of d<sup>8</sup> ions (Douglas et al., 1983). The  $\Delta H_d$  data for the corresponding azurin derivatives are also presented in Figure 7 and as would be expected, the Ni(II) adduct is the least stable. The most striking result in Figure 7 is that in comparison to the bromide results, the Cu(II) derivative of azurin is considerably stabilized relative to the Zn(II) form. The particular low symmetry

structure of the metal binding site in azurin may be responsible. Unlike Zn(II), Cu(II) achieves some stabilization by virtue of a distortion from idealized tetrahedral symmetry, because of the (pseudo) Jahn–Teller effect.

The fact that particular distortions from tetrahedral symmetry favor the binding of Cu(II) over Zn(II) may have physiological significance because zinc tends to be more abundant in cells than copper. Williams has estimated that the intracellular concentration of Zn(II) is 10<sup>3</sup> that of Cu(II) (Williams, 1983). That zinc proteins from other organisms, e.g., mammalian liver alcohol dehydrogenase (Branden, 1975), present donor sets very much like that of azurin underscores the possibility that different metal ions can compete for a given binding site. Parenthetically, we may note that zinc binding sites in alcohol dehydrogenase and other proteins also frequently exhibit low-symmetry coordination geometries (Galdes & Vallee, 1983), geometries which may favor the binding of Cu(II). Indeed, in carbonic anhydrase, the binding of Cu(II) is favored over Zn(II) at pH 5.5 (Lindsog & Nyman, 1965). Of course, the higher intracellular concentration of zinc will tend to balance this effect.

It is fortunate that the intracellular levels of Hg(II) are quite low because the enthalpy data suggest that azurin binds Hg(II) even better than Cu(II). This is reasonable because the low effective coordination number is well suited to the binding of Hg(II) (Grdenik, 1965) and of course Hg(II) has a great affinity for thiol groups. In another study, we have taken advantage of this affinity in preparing a mixed-metal derivative of laccase in which Hg(II) resides in the type 1 binding site (Morie-Bebel et al., 1984).

Perhaps the biggest surprise in Table I is the relatively low  $\Delta H_d$  observed for Ag<sup>I</sup>Az. In the case of plastocyanin, the addition of Ag(I) or Hg(II) causes partial bleaching of the blue color (Kato & Takamiya, 1964), and this suggests that the Ag(I) and Cu(II) derivatives of plastocyanin have comparable stabilities.

**Metal Titrations.** Previous studies of azurin derivatives have focused on the 1:1 adducts in which metal ions replace the copper center of the native protein. The results in Figure 5 suggest that another species forms when the Hg(II) to protein ratio is less than 1:1 and that it achieves a maximum concentration when the ratio is about 1:2. The stoichiometry suggests that a protein dimer is involved, and this interpretation is supported by size-exclusion chromatography. Both apoazurin and Hg<sup>II</sup>Az elute from a Sephadex G-75 column with the same retention time as native azurin. However, a sample which contains apoazurin and 0.5 equiv of Hg(II) exhibits a shorter retention time, consistent with a higher effective molecular weight (Figure 8). Any discussion of the dimer structure would be speculative, but since the dimerization is not observed in the apoprotein, it seems evident that mercury is required to bridge the protein molecules. Moreover, the stability of the dimer must depend on surface interactions which occur near the point(s) at which the proteins contact each other. Favorable interactions of this type are possible because in the crystalline state the asymmetric unit consists of a cluster of four molecules among which a number of surface to surface contacts are observed (Adman et al., 1978). Mercury has previously been observed to induce dimers of serum albumin, possibly via the coordination of protein thiols (Sarker, 1983). Some structural reorganization would be required in order for Hg(II) to bridge between the cysteine sulfurs of the respective type 1 binding sites. Despite the fact that the mercury environment is likely to be quite different in the dimer than it is in Hg<sup>II</sup>Az, the enthalpies of denaturation

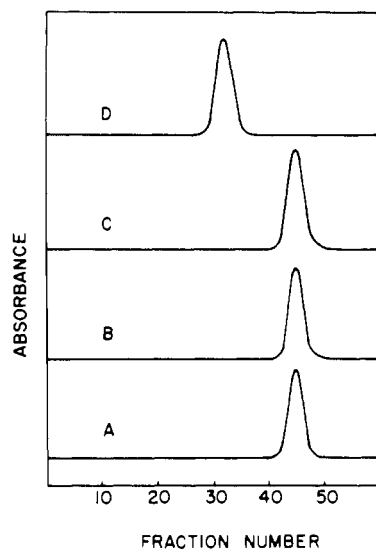


FIGURE 8: Elution profiles of azurin derivatives. (A) Apoazurin; (B)  $\text{Cu}^{\text{II}}\text{Az}$ ; (C)  $\text{Hg}^{\text{II}}\text{Az}$ ; (D) apoazurin and 0.5 equiv of  $\text{Hg}(\text{II})$ . The buffer was 0.02 M phosphate, pH 8.0, and the temperature was 21 °C. The protein absorbance was monitored at 280 nm.

are very similar (Figure 5). This observation underscores the difficulties in interpreting enthalpy data in such complex systems.

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#### REFERENCES

- Addison, A. W., Rao, T. N., & Sinn, E. (1984) *Inorg. Chem.* 23, 1957-1967.
- Adman, E. T. (1985) *Top. Mol. Struct. Biol.* 6, 1-42.
- Adman, E. T., & Jensen, L. H. (1981) *Isr. J. Chem.* 21, 8-12.
- Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol.* 123, 35-47.
- Ambler, R. P., & Brown, L. H. (1967) *Biochem. J.* 104, 784-825.
- Anzenbacher, P., Hudeček, J., & Stružinsky, R. (1982) *FEBS Lett.* 149, 208-211.
- Bencini, A., & Gatteschi, D. (1983) *J. Am. Chem. Soc.* 105, 5535-5541.
- Bianchi, A., & Paoletti, P. (1982) *Inorg. Chim. Acta* 96, L37-L40.
- Blaszak, J. A., Ulrich, E. L., Markley, J. L., & McMillin, D. R. (1982) *Biochemistry* 21, 6253-6258.
- Blaszak, J. A., McMillin, D. R., Thorton, A. T., & Tennent, D. L. (1983) *J. Biol. Chem.* 258, 9886-9892.
- Boyer, P. D. (1954) *J. Am. Chem. Soc.* 76, 4331-4337.
- Brändén, C. I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes (3rd Ed.)* 11, 103-190.
- Brill, A. S. (1977) *Mol. Biol., Biochem. Biophys.* 26, 40-80.
- Canthers, G. W., Hill, H. A. O., Kitchen, N. A., & Adman, E. T. (1984) *Eur. J. Biochem.* 138, 141-152.
- Chlebowski, J. F., & Mabrey, S. (1977) *J. Biol. Chem.* 252, 7042-7052.
- Coleman, J. E., & Chlebowski, J. F. (1979) *Adv. Inorg. Biochem.* 1, 1-67.
- Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London)* 272, 319-324.
- Donovan, J. W. (1984) *Trends Biochem. Sci. (Pers. Ed.)* 9, 340-344.
- Douglas, B., McDaniel, D. H., & Alexander, J. J. (1983) *Concepts and Models of Inorganic Chemistry*, 2nd ed., pp 268-277, Wiley, New York.
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* 82, 70-77.
- Engeseth, H. R., McMillin, D. R., & Otvos, J. D. (1984) *J. Biol. Chem.* 259, 4822-4826.
- Farver, O., & Pecht, I. (1981) *Met. Ions Biol. Syst.* 3, 151-192.
- Farver, O., & Pecht, I. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 1, Chapter 7, CRC Press, Boca Raton, FL.
- Fee, J. A. (1975) *Struct. Bonding (Berlin)* 23, 1-60.
- Ferris, N. S., Woodruff, W. H., Tennent, D. L., & McMillin, D. R. (1984) *J. Am. Chem. Soc.* 106, 3677-3678.
- Freeman, H. C. (1981) *Coord. Chem.* 21, 29-51.
- Galdes, A., & Vallee, B. L. (1983) *Met. Ions Biol. Syst.* 15, 1-54.
- Gray, H. B., & Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) Vol. 1, pp 1-39, Wiley, New York.
- Gray, H. B., & Malmström, B. G. (1983) *Comments Inorg. Chem.* 2, 203-209.
- Grdenik, D. (1965) *Q. Rev., Chem. Soc.* 19, 303-328.
- Gus, J. M., & Freeman, H. C. (1983) *J. Mol. Biol.* 169, 521-563.
- Hill, H. A. O., & Smith, B. E. (1976) *Biochem. Biophys. Res. Commun.* 70, 783-790.
- Hill, H. A. O., & Smith, B. E. (1979) *J. Inorg. Biochem.* 11, 79-93.
- Holwerda, R. A., Wherland, S., & Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng.* 5, 363-396.
- Katoh, S., & Takamiya, A. (1964) *J. Biochem. (Tokyo)* 55, 378-387.
- Lindskog, S., & Nyman, P. O. (1964) *Biochim. Biophys. Acta* 85, 462-474.
- Lowry, O. H., Rosenbrough, N. J., Fan, A. L., & Rendall, R. J. (1951) *J. Biol. Chem.* 193, 265-275.
- Malmström, B. G., & Vänngård, T. J. (1960) *J. Mol. Biol.* 2, 118-124.
- Markley, J. L., Ulrich, E. L., Berg, S. P., & Krogmann, D. W. (1975) *Biochemistry* 14, 4428-4433.
- McMillin, D. R., & Morris, M. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 6567-6570.
- McMillin, D. R., & Engeseth, H. R. (1986) in *Biological and Inorganic Copper Chemistry* (Karlan, K. D., & Zubieta, J., Eds.) Vol. I, pp 1-10, Adenine Press, Guilderland, NY.
- McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 4760-4762.
- Morie-Bebel, M. M., Morris, M. C., Menzie, J. L., & McMillin, D. R. (1984) *J. Am. Chem. Soc.* 106, 3677-3678.
- Musci, G., Desideri, A., Morpurgo, L., & Tosi, L. (1985) *J. Inorg. Biochem.* 23, 93-102.
- Norris, G. E., Anderson, B. F., & Baker, E. N. (1983) *J. Mol. Biol.* 165, 501-521.
- Penfield, K. W., Gewirth, A. A., & Solomon, E. I. (1985) *J. Am. Chem. Soc.* 107, 4519-4529.
- Privalov, P. L. (1982) *Adv. Protein Chem.* 35, 1-103.
- Privalov, P. L., Mateo, P. L., Khechivashvili, N. N., Stepanov, V. M., & Revina, L. P. (1981) *J. Mol. Biol.* 152, 445-464.
- Reinhammar, B. (1970) *Biochim. Biophys. Acta* 205, 35-47.
- Richardson, J. S. (1984) in *The Protein Folding Problem* (Wetlaufer, D. B., Ed.) pp 1-28, Westview Press, Boulder, CO.

- Sakena, V. P., & Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 969-972.
- Sarkar, B. (1983) *Life Chem. Rep.* 1, 165-207.
- Takagi, T., & Isemura, T. (1964) *J. Biochem. (Tokyo)* 56, 344-350.
- Tang, S. P. W., Coleman, J. E., & Myer, Y. P. (1968) *J. Biol. Chem.* 243, 4286-4297.
- Tennent, D. L., & McMillin, D. R. (1979) *J. Am. Chem. Soc.* 101, 2307-2311.
- Williams, R. J. P. (1983) *Pure Appl. Chem.* 55, 35-46.

## Kinetics of Intracellular Degradation of Newly Synthesized Collagen<sup>†</sup>

Robert S. Bienkowski\*

*Pulmonary Division, Department of Pediatrics, Schneider Children's Hospital of Long Island Jewish Medical Center, New Hyde Park, New York 11042*

Samantha F. Curran and Richard A. Berg

*Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Rutgers Medical School, Piscataway, New Jersey 08854*

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**ABSTRACT:** The objective of this work was to determine the time dependence of the basal component of intracellular degradation of newly synthesized collagen. Chick embryo tendon fibroblasts were incubated with [<sup>14</sup>C]proline, and degradation was quantified by measuring hydroxy[<sup>14</sup>C]proline in a low molecular weight fraction. When cultures were pulse labeled for 15 min and then incubated under chase conditions for 105 min, the amount of degraded collagen attained a value equal to approximately 20% of the amount synthesized during the labeling period; the data were fit with a simple exponential function that had a 40-min rise time and a 12-min lag time. In continuously labeled cultures, the rates of collagen synthesis and secretion reached constant values within 15 and 45 min, respectively. Degradation products were first detected 6-9 min after collagen synthesis began and were transported out of the cells more rapidly than intact collagenous molecules; however, percent degradation increased slowly and did not reach a constant value even after 240 min of incubation. Since collagen degradation lags collagen synthesis, it follows that degradation is a posttranslational, rather than a cotranslational, process, and since degradation and secretion are kinetically distinguishable, it follows that they occur in parallel pathways. A simple nonlinear model for posttranslational processing of collagen is proposed.

Although many secretory proteins are catabolized soon after being synthesized rather than transported intact to the extracellular space (Bienkowski, 1983), rapid intracellular degradation has been documented most extensively for collagen synthesized by connective tissue cells in culture (Rennard et al., 1982; Bienkowski, 1984a). Recent work has shown that catabolism of newly synthesized collagen can proceed by at least two different pathways (Berg et al., 1980; Bienkowski, 1984a). One of these, the basal pathway, accounts for the breakdown of 10-20% of all newly synthesized collagen (Bienkowski, 1984b). Another pathway is activated when structurally abnormal collagen is synthesized; abnormalities in molecular structure can be caused by depriving cultures of ascorbate or by adding a proline analogue to the incubation medium. Several lines of evidence demonstrate that enhanced degradation is mediated by lysosomal proteases and occurs in lysosomes (Berg et al., 1980, 1984; Bienkowski, 1984b). In contrast, little is known about the mechanism of basal degradation. A complete description of this pathway would require identification of its constituent proteases and substrates, determination of its location within the cell, and measurement of the time scale on which it operates relative to synthesis and posttranslational processing of collagen.

The specific questions we wished to address in the present study were the following: When does basal degradation begin, and what is the time dependence for the generation of degradation products? We used matrix-free tendon fibroblasts from chick embryos because a large percentage of the protein synthetic activity in these cells is devoted to collagen and because the kinetics for the synthesis, posttranslational modification, and secretion of collagen have been established for these cells (Dehm & Prockop, 1972; Harwood et al., 1976; Kao et al., 1977, 1979). Our data show that, in comparison to synthesis and secretion of collagen, intracellular degradation is a very slow process.

### EXPERIMENTAL PROCEDURES

**Materials.** Radioactive isotopes were purchased from New England Nuclear, Boston, MA. L-[U-<sup>14</sup>C]Proline (approximate specific activity 280 mCi/mol) had a stated radiochemical purity >99%, and this was verified in our laboratory. *trans*-4-Hydroxy-L-[G-<sup>3</sup>H]proline (specific activity 5.4 Ci/mol) was purified by ion-exchange chromatography as described (Bienkowski & Engels, 1981). Purified bacterial collagenase (form III) was purchased from Advance Biofactures, Lynbrook, NY.

**Incubation Protocols.** Tendon fibroblasts were isolated from 17-day-old chick embryos according to the method of Dehm and Prockop (1972) as modified by Kao et al. (1977). The cells were suspended at a density of approximately 10<sup>7</sup>/mL

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\* Author to whom correspondence should be addressed.