Binding of Imidazoles to Manganese(III) Protoporphyrin in Water†

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ABSTRACT: Equilibrium constants for the addition of water to manganese(III) protoporphyrin in acetone were measured at 21 °C by the visible absorption method. The binding constants of the first and second water molecules were determined to be 209 ± 3 M^{-1} and 22 ± 1 M^{-1}, respectively. This observation suggests that in water the fifth and sixth coordination positions of manganese(III) protoporphyrin are coordinated by two water molecules and that manganese(III) protoporphyrin is monomeric. Addition of 1-methylimidazole and 2-methylimidazole to manganese(III) protoporphyrin in water was also investigated spectrophotometrically, and the respective monoimidazole adducts were found to be born in appreciable amounts. The binding constants of the first imidazole were 24.7 ± 1.0 M^{-1} for 1-methylimidazole and 16.5 ± 0.9 M^{-1} for 2-methylimidazole in 0.1 M tris(hydroxymethyl)aminomethane buffer at pH 8.0 and 21 °C. The steric effect of the 2-methyl group in the aquomanganese(III) (2-methylimidazole) species is expected to mimic the proximal strain in the α subunits of human adult manganese(III) hemoglobin. The energy associated with the strain in the Mn(III)–imidazole bond of the model complex was estimated to be 240 cal/mol. The small strain energy in the Mn(III)–imidazole bond shows that the Mn(III)–imidazole bond is easily tensed by a slight energy and implies that the major part of the quaternary transition energy in manganese(III) hemoglobin by inositol hexaphosphate binding is widely delocalized around the globin moiety, as has been proposed for hemoglobin.

The change of the oxygen affinity of hemoglobin involves a switch in the conformation at some fraction of oxygenation from the T (tensed) to the R (relaxed) quaternary structures. The molecular details of this conformational change have been reported by Perutz on the basis of the X-ray data (Perutz, 1970). In his model, the heme iron movement from the out-of-plane position of porphyrin in the deoxy state to the in-plane position in the oxy state was suggested to induce the quaternary transition. He and his co-workers (Perutz et al., 1974, 1978) extended the trigger mechanism to iron(III) hemoglobin to support the idea linking the spin state of heme iron and the quaternary conformation. Recent X-ray analysis (Fermi & Perutz, 1977) showed that human iron(III) hemoglobin fluoride with the allosteric effector inositol hexaphosphate is indeed in the T state. On the basis of the proposal by Perutz, the interaction between the heme iron and the proximal base in the model porphyrin complexes has been investigated by a number of investigators (Jameson et al., 1980; Spiro et al., 1979; Traylor & Berzinis, 1980; Scheidt, 1977).

There has been considerable interest in understanding the role of iron in hemoglobin by examining the structural and functional changes produced by the replacement of iron by other metal ions (Ikeda-Saito & Yonetani, 1980; Gibson & Hoffman, 1979; Dickinson, 1976). In this work, we have attempted to model and study the monoadducts of 1-methylimidazole and 2-methylimidazole to manganese(III) protoporphyrin in water to gain an insight into the Mn(III)–imidazole bond and to compare the result with the protein system, manganese(III) hemoglobin (Hoffman et al., 1975). The model complex study with manganese(III) protoporphyrin provides a unique opportunity to examine the formation and the property of the monoimidazole adduct, which was found to be accumulated in appreciable amounts in the equilibrium mixture, while the monoimidazole adduct is scarcely accumulated in iron(III) porphyrin complexes (Pasternack et al., 1978; Walker et al., 1976; Coyle et al., 1973; Morishima et al., 1980). We analyze here the energy difference associated with the proximal tension in the model complexes and refer to the implication on the quaternary transition in human manganese(III) hemoglobin.

Materials and Methods

Chemicals. Protoporphyrin was obtained from Sigma, and 1-methylimidazole and 2-methylimidazole were from Nakarai Chemicals Ltd. (Kyoto). 2-Methylimidazole was recrystallized 2 times from benzene followed by vacuum drying. 1-Methylimidazole was distilled from zinc dust and then from potassium hydroxide (Pasternack et al., 1978). All other chemicals were reagent grade.

Preparation of Manganese(III) Protoporphyrin Chloride. Manganese(III) protoporphyrin chloride was prepared and purified by chromatography according to the method of Yonetani & Asakura (1969). The purity of the sample was checked by visible spectral comparison with the authentic one reported by Waterman & Yonetani (1970).

Spectral Measurements. The visible absorption spectra were obtained on a Union-Giken Model SM-401 spectrometer. The ligand binding constant at 21 °C was obtained by using quartz cells with a path length of 1 cm. A 4-mL sample of an approximately 10 μM solution of manganese(III) protoporphyrin in acetone or water (0.1 M Tris[1] buffer, pH 8.0) was titrated with 1–4-μL aliquots of ligand stock solutions. Measurements were made between 460 and 480 nm, where the largest absorption changes upon ligand addition were observed.

Data Analysis. The equilibrium constants of ligand binding were determined by the χ^2 curve-fitting routine, CHISQ, programmed in a Nicolet 1080 computer.

Results and Analyses

(*A*) *Coordination of Water.* The visible spectra of manganese(III) protoporphyrin in acetone revealed different isosbestic points at high and low water concentrations. This observation may be rationalized by the stepwise equilibria described by eq 1 and 2 where P is manganese(III) proto-

$$P + H_2O \overset{K_1}{\rightleftharpoons} P{\cdot}H_2O \quad (1)$$

$$P{\cdot}H_2O + H_2O \overset{K_2}{\rightleftharpoons} P{\cdot}(H_2O)_2 \quad (2)$$

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[1] Abbreviation used: Tris, tris(hydroxymethyl)aminomethane.

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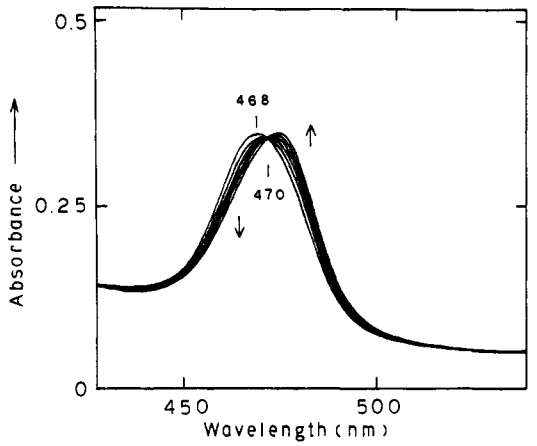

FIGURE 1: Visible absorption changes upon addition of small amounts of water to manganese(III) protoporphyrin in acetone at 21 °C. Arrows show the increase or decrease of the absorption.

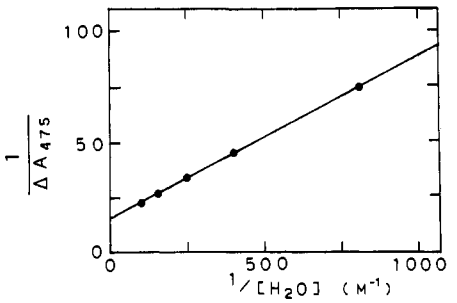

FIGURE 2: Relation between absorbance and water concentration. Ordinate, reciprocal of absorbance; abscissa, reciprocal of water concentration in M^{-1}. Intercept = 15.11 ± 0.18, and slope = (7.25 ± 0.08) × 10^{-2} M.

porphyrin, K_1 = [P·H_2O]/[P][H_2O], and K_2 = [P·$(H_2O)_2$]/[P·H_2O][H_2O].

(*i*) *Coordination of the First Water.* Addition of small amounts of water to manganese(III) protoporphyrin in acetone produced changes in the visible absorption bands with a clear isosbestic point at 470 nm (Figure 1). The spectral changes at 475 nm were analyzed according to eq 3 where A_0 and A_1

$$\frac{1}{A - A_0} = \frac{1}{K_1(A_1 - A_0)} \frac{1}{[H_2O]} + \frac{1}{A_1 - A_0} \quad (3)$$

are the absorbances of manganese(III) protoporphyrin and aquomanganese(III) protoporphyrin respectively, and A, the observed absorbance, is the weighted average of A_0 and A_1. A plot of $1/(A - A_0)$ vs. $1/[H_2O]$ of Figure 1 as evaluated from eq 3 is shown in Figure 2. The linear relationship confirms the equilibrium scheme of eq 1. The formation constant of the mono-water-coordinated species, K_1 = 209 ± 3 M^{-1}, was obtained.

(*ii*) *Coordination of the Second Water.* Further addition of water to manganese(III) protoporphyrin in acetone broke the isosbestic point in Figure 2 due to the overlap of the new equilibrium presented by eq 2 (Figure 3). The precise analysis for the equilibrium of eq 2 according to eq 3 was not possible. Such overlapping equilibria may be evaluated from the relationship (Rossotti & Rossotti, 1961)

$$A = \frac{A_0 + A_1K_1[H_2O] + A_2K_1K_2[H_2O]^2}{1 + K_1[H_2O] + K_1K_2[H_2O]^2} \quad (4)$$

where A_i's (i = 0, 1, and 2) are the absorbances of manganese(III) protoporphyrin coordinated by i water molecule(s), and A, the observed absorbance, is the weighted average of

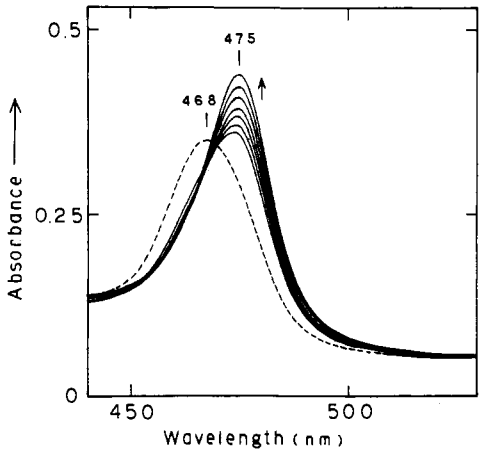

FIGURE 3: Visible absorption changes upon addition of water to manganese(III) protoporphyrin in acetone at 21 °C. The dotted curve shows the spectrum when water is absent.

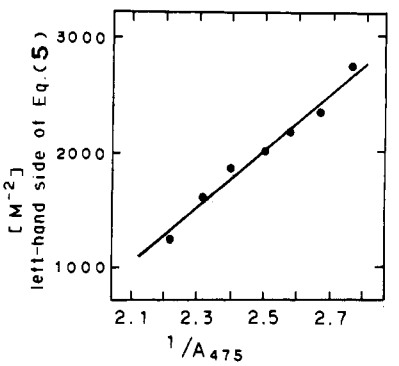

FIGURE 4: Analysis of the absorption data in Figure 3 according to eq 5. Intercept = −4494 ± 199 M^{-2}, and slope = (84.9 ± 0.4) × 10^4 M^{-2}.

A_i's. From the values of K_1 and A_1 determined above, K_2 and A_2 may be determined by rearranging eq 4 into eq 5 (Rossotti

$$\frac{A - A_0}{A[H_2O]^2} + \frac{(A - A_1)K_1}{A[H_2O]} = K_1K_2A_2\frac{1}{A} - K_1K_2 \quad (5)$$

& Rossotti, 1961). The values of $K_1K_2A_2$ and $-K_1K_2$ are determined as the slope and intercept, respectively, of the linear plot of the left-hand side of eq 5 against $1/A$. Such a plot is shown in Figure 4 for the absorption changes at 475 nm in Figure 3. From the least-squares analysis of the result in Figure 4, K_2 = 22 ± 1 M^{-1} was obtained.

(*B*) *Coordination of 1-Methylimidazole and 2-Methylimidazole to Manganese(III) Protoporphyrin in Water.* Binding of imidazole to manganese(III) protoporphyrin may be described by eq 6 and 7 where P is manganese(III) pro-

$$P + Im \rightleftharpoons P{\cdot}Im \quad (6)$$

$$P{\cdot}Im + Im \rightleftharpoons P{\cdot}(Im)_2 \quad (7)$$

toporphyrin (axial ligands are not shown), Im is 1-methyl- or 2-methylimidazole, K_1 = [P·Im]/[P][Im], and K_2 = [P·$(Im)_2$]/[P·Im][Im]. Coordination of the first imidazole may be analyzed by use of the linear equation with [H_2O] substituted by [Im] in eq 3. Upon further addition of 1-methylimidazole in the 0.1–1 M range, the isosbestic point first observed at 476 nm shifted to 472 nm (Figure 5). The spectral change in Figure 5 is consistent with the stepwise equilibria described by eq 6 and 7.

Although the two equilibria overlap somewhat, it was possible to observe only the first coordination of imidazole by

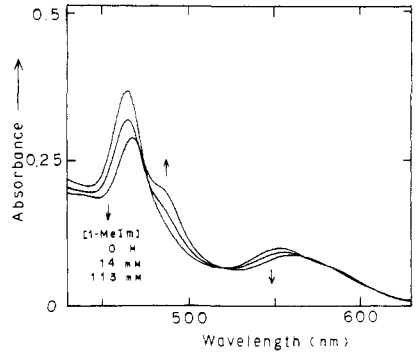

FIGURE 5: Visible spectra of manganese(III) protoporphyrin at the three different 1-methylimidazole concentrations in 0.1 M Tris buffer at pH 8.0 and 21 °C. Lack of the isosbestic point at about 470 nm is noted.

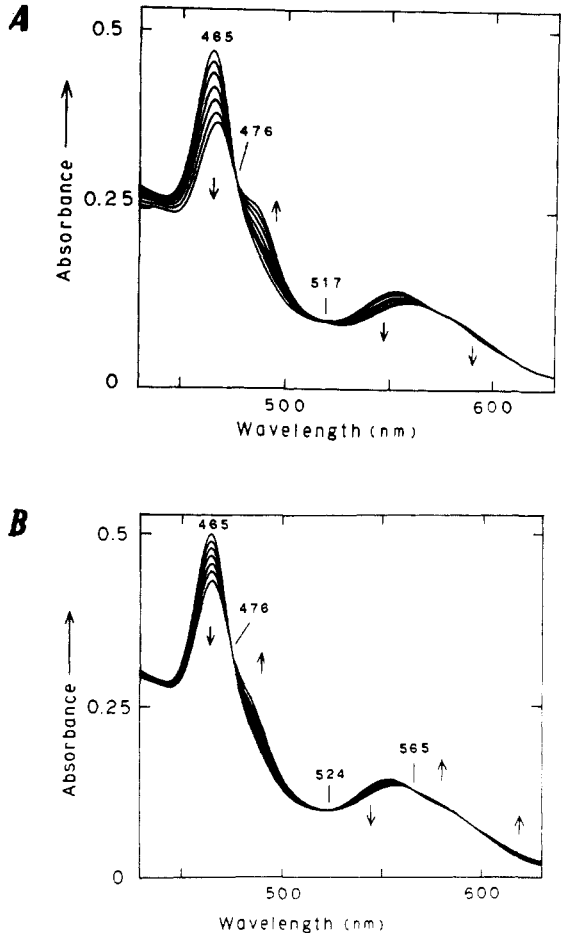

FIGURE 6: Visible spectral changes upon addition of (A) 1-methylimidazole and (B) 2-methylimidazole to manganese(III) protoporphyrin in 0.1 M Tris buffer at pH 8.0 and 21 °C.

careful addition of ligand in small amounts. Figure 6 shows the spectra of manganese(III) protoporphyrin titration with 1-methylimidazole or 2-methylimidazole in 0.1 M Tris buffer, pH 8.0. The existence of only two absorbing species is substantiated by the presence of the clear isosbestic points at 476 and 517 nm for 1-methylimidazole binding and at 476, 524, and 565 nm for 2-methylimidazole binding. Plots of $1/(A - A_0)$ at 465 nm vs. $1/[\mathrm{Im}]$ are shown in Figure 7 with the lines representing the least-squares results. Binding constants of $K_1 = 24.7 \pm 1.0\ \mathrm{M}^{-1}$ for 1-methylimidazole and $K_1 = 16.5 \pm 0.9\ \mathrm{M}^{-1}$ for 2-methylimidazole were obtained to eq 6.

Discussion

Formation of Aquomanganese(III) Protoporphyrin.

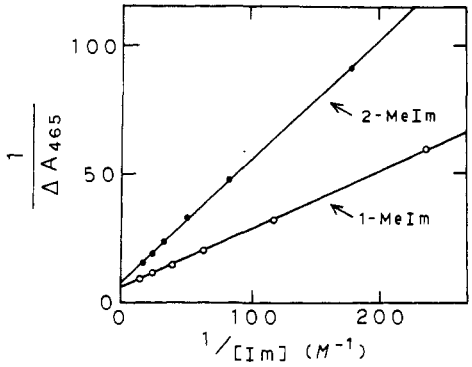

FIGURE 7: Relation between absorbance and imidazole concentration. (○) 1-Methylimidazole, intercept = 5.67 ± 0.22 and slope = (2.37 ± 0.04) × 10^{-1} M; (●) 2-methylimidazole, intercept = 7.77 ± 0.42 and slope = (4.71 ± 0.06) × 10^{-1} M.

Manganese(III) porphyrins are known to be soluble in chloroform, acetic acid, and water (Yonetani & Asakura, 1969; Waterman & Yonetani, 1970; Loach & Calvin, 1963; Boucher, 1970). However, whether these solvent molecules can coordinate to manganese(III) porphyrins or not was unclear. Boucher (1972) suggested that the elemental analysis of the acetate salts of manganese(III) porphyrins can equally be fitted by assuming a $H_2O{\cdot}OH^-$ complex and that the only species isolated from aqueous solution in the absence of coordinating anion is the hydroxide complex. Although several authors have assumed the $H_2O{\cdot}Cl^-$ structure for manganese(III) porphyrins in chloroform (Loach & Calvin, 1963; Boucher, 1970; Janson et al., 1973), the X-ray anslysis by Tulinsky & Chien (1977) revealed that the crystal of manganese(III) tetraphenylporphyrin chloride grown from noncoordinating acetone is 5-coordinate without additional water at the sixth coordination site.

For manganese(III) porphyrin dissolved in water, the association state and axial ligand have not been identified. The presence of the dimer, $H_2O{\cdot}Mn(III){-}O{-}Mn(III){\cdot}H_2O$, was alluded for manganese(III) tetraphenylporphyrin by Boucher (1973). Loach & Calvin (1963) assumed for manganese(III) hematoporphyrin in water at neutral pH that water and one of the propionic residues on the porphyrin ring coordinate to Mn(III). Boucher (1972) had tentatively assigned the coordination of water and hydroxide to manganese(III) porphyrins dissolved in water at a neutral pH. The present experiment of water titration to manganese(III) protoporphyrin in acetone shows that 2 mol of water coordinate to 1 mol of manganese(III) protoporphyrin, contrary to the earlier suggestions. The first ligation of water with $K_1 = 209\ \mathrm{M}^{-1}$ in acetone is the direct coordination of water to Mn(III), because the sixth position of a similar compound, manganese(III) tetraphenylporphyrin chloride, is vacant (Tulinsky & Chien, 1977). The second ligation of water with $K_2 = 22\ \mathrm{M}^{-1}$ is the displacement of Cl^- at the fifth position by the water. This picture may be supported by the smallness of K_2 compared with K_1. It is thus likely that the axial positions of manganese(III) porphyrins in water are coordinated by two water molecules and that diaquomanganese(III) protoporphyrin is monomeric, at least in the concentration range examined here, i.e., [porphyrin] = 8–10 μM. The present result might not be suprising in view of the coordinating property of water to the heme (Scheidt et al., 1979) and hemoprotein (Ladner et al., 1977) and of the high concentration of water, as much as 55.5 M. It is to be noted that the visible absorption maximum of diaquomanganese(III) protoporphyrin is observed at 475 nm in acetone (Figure 3) and at 465 nm in water (Figure 6). The absorption around 475 nm of manganese(III) porphyrin was

assigned to the charge transfer band from porphyrin π orbitals to Mn(III) d orbitals, and the solvent which is the electron donor could shift this band to the shorter wavelength (Boucher, 1970; Hill et al., 1967). The blue shift of the charge transfer band by 10 nm in going from acetone to water solvents suggests that the coordinating water molecules could interact with other water molecules in the nearest solvent sphere to affect the manganese(III) prophyrin interaction through the H_2O–Mn-(III)–OH_2 bonds.

Formation of the Mixed-Ligand Complexes. On the basis of the above result of water binding to manganese(III) protoporphyrin in acetone, it is very likely that the axial positions of manganese(III) protporphyrin in water are occupied by two water molecules. The spectral changes in Figure 6 upon addition of 1-methylimidazole or 2-methylimidazole are thus attributable to the formation of the mixed-ligand complex aquomanganese(III) protoporphyrin 1-methyl- or 2-methylimidazole. The possibility of the bisimidazole adduct formation is readily distinguished by the gradual shift of the isosbestic point initially observed at 476 nm to 472 nm upon further addition of 1-methylimidazole (Figure 5). The accumulation of the monoimidazole adducts in appreciable amounts in manganese(III) protoporphyrin is in contrast with the results of iron(III) porphyrins. Although the formation of the monoimidazole adduct of the hindered imidazole such as 2-methylimidazole or benzimidazole in iron(III) octaethylporphyrin (Ogoshi et al., 1980; Morshima et al., 1980) or iron(III) protoporphyrin dimethyl ester (Yoshimura & Ozaki, 1979) is known, the monoimidazole adduct of the nonhindered imidazole or 1-methylimidazole is hardly accumulated (Pasternack et al., 1978; Walker et al., 1976; Coyle et al., 1973).

Implication to the Quaternary Structural Change in Manganese(III) Hemoglobin. Moffat et al. (1974) reported that manganese(III) hemoglobin, in which Fe(III) is replaced by Mn(III), has the same quaternary structure as iron (III) hemoglobin. Addition of the allosteric effector, inositol hexaphosphate, produced the ultraviolet difference spectrum similar to that produced in iron(III) hemoglobin, suggesting the presence of the T quaternary structure in manganese(III) hemoglobin (Hoffman et al, 1975). Manganese(II) hemoglobin is also known to display the allosteric effect in the proton affinity in NO binding and the redox properties (Hoffman et al., 1975; Gibson & Hoffman, 1979).

The 6-coordinate compound, aquo(1-methylimidazole)- or aquo(2-methylimidazole)manganese(III) protoporphyrin, is expected to serve as the prosthetic model for the α subunits in manganese(III) hemoglobin, for which the X-ray analysis by Moffat et al. (1976) showed that the α subunits have a bound water, while the β subunits do not.

According to the hypothesis linking the quaternary structure and the spin state of the heme iron (Perutz, 1970), special attention on the proximal F8 histidine has been paid to account for the ligand affinities and spectral properties of hemoglobin. In the present model complex, aquomanganese(III) 2-methylimidazole, the Mn(III)–imidazole linkage is thought to be under tension in the form of tilting and elongation due to the steric effect of the 2-methyl group. It is interesting to estimate the energy needed for going from the unstrained Mn(III)–1–methylimidazole bond to the strained Mn(III)–2–methylimidazole bond. Since the association rates of the similar types of imidazoles are approximated to be the same, the difference in the formation constants of the two mixed-ligand complexes is thought to reflect the different dissociation rates of the two imidazoles. Thus, the changes in the free energy associated with the steric effect of the 2-methyl group can be calculated by subtracting the ligation free energy of 2-methylimidazole from that of 1-methylimidazole. The difference in the free energy is estimated at 21 °C as $\Delta(\Delta G) = RT \ln (24.7/16.5) \approx 240$ cal/mol. This energy difference is solely due to the steric effect of the 2-methyl group, because the basicities of the imidazoles are almost the same ($pK = 7.33$ for 1-methylimidazole and $pK = 7.56$ for 2-methylimidazole; Albert, 1963). The value of 240 cal/mol obtained for manganese(III) protoporphyrin is comparable with 300 cal/mol obtained from the near-infrared analysis for the tension in iron(II) deoxyhemoglobin and iron(III) hemoglobin fluoride by Perutz et al. (1974). Unfortunately, the free energy of the allosteric transition in manganese(III) hemoglobin has not been determined yet. However, the present analysis on the model complexes shows that the Mn(III)–imidazole bond is easily restrained by a rather small energy and implies that a large fraction of the free energy of the allosteric transition in manganese(III) hemoglobin (Hoffman et al., 1975) is widely delocalized around the globin moiety as has been proposed for iron(II) hemoglobin (Drago et al., 1978; Hopfield, 1973).

Acknowledgments

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References

Albert, A. (1963) *Phys. Methods Heterocycl. Chem. 1*, 1.
Boucher, L. J. (1970) *J. Am. Chem. Soc. 92*, 2725–2730.
Boucher, L. J. (1972) *Coord. Chem. Rev. 7*, 289–329.
Boucher, L. J. (1973) *Ann. N.Y. Acad. Sci. 206*, 409–419.
Coyle, C. L., Rafson, P. A., & Abbott, E. H. (1973) *Inorg. Chem. 12*, 2007–2010.
Dickinson, L. C. (1976) *J. Chem. Educ. 53*, 381–385.
Drago, R. S., Beugelsdijk, T., Breese, J. A., & Cannady, J. P. (1978) *J. Am. Chem. Soc. 100*, 5374–5382.
Fermi, G., & Perutz, M. F. (1977) *J. Mol. Biol. 114*, 421–431.
Gibson, Q. H., & Hoffman, B. M. (1979) *J. Biol. Chem. 254*, 4691–4697.
Hill, H. A. O., Macfarlane, A. J., & Williams, R. J. P. (1967) *Chem. Commun.*, 905–906.
Hoffman, B. M., Gibson, Q. H., Bull, C., Crepau, R. H., Edelstein, S. J., Fisher, R. G., & McDonald, M. J. (1975) *Ann. N.Y. Acad. Sci. 244*, 174–186.
Hopfield, J. J. (1973) *J. Mol. Biol. 77*, 207–222.
Ikeda-Saito, M., & Yonetani, T. (1980) *J. Mol. Biol. 138*, 845–858.
Jameson, B. J., Molinaro, F. S., Ibers, J. A., Collman, J. P., Brauman, J. I., Rose, E., & Suslick, K. S. (1980) *J. Am. Chem. Soc. 102*, 3224–3237.
Janson, T. R., Boucher, L. J., & Katz, J. J. (1973) *Inorg. Chem. 12*, 940–943.
Ladner, R. C., Heidner, E. J., & Perutz, M. F. (1977) *J. Mol. Biol. 114*, 385–414.
Loach, P. A., & Calvin, M. (1963) *Biochemistry 2*, 361–371.
Moffat, K., Loe, R. S., & Hoffman, B. M. (1974) *J. Am. Chem. Soc. 96*, 5259–5261.
Moffat, K., Loe, R. S., & Hoffman, B. M. (1976) *J. Mol. Biol. 104*, 669–685.
Morishima, I., Kitagawa, S., Matsuki, E., & Inubushi, T. (1980) *J. Am. Chem. Soc. 102*, 2429–2437.
Ogoshi, H., Sugimoto, H., & Yoshida, Z. (1980) *Biochim. Biophys. Acta 621*, 19–28.
Pasternack, R. F., Gillies, B. S., & Stahlbush, J. R. (1978) *J. Am. Chem. Soc. 100*, 2613–2619.
Perutz, M. F. (1970) *Nature (London) 228*, 726–739.

Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., & Slade, E. F. (1974) *Biochemistry 13*, 2187–2200.
Perutz, M. F., Sanders, J. K. M., Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W.-M., Ho, C., Giannini, I., Pörschke, D., & Winkler, H. (1978) *Biochemistry 17*, 3640–3652.
Rossotti, F. J. C., & Rossotti, H. (1961) *The Determination of Stability Constants*, p 277, McGraw-Hill, New York.
Scheidt, W. R. (1977) *Acc. Chem. Res. 10*, 339–345.
Scheidt, W. R., Cohen, I. A., & Kastner, M. E. (1979) *Biochemistry 18*, 3546–3552.
Spiro, T. G., Stong, J. D., & Stein, P. (1979) *J. Am. Chem. Soc. 101*, 2648–2655.
Traylor, T. G., & Berzinis, A. P. (1980) *J. Am. Chem. Soc. 102*, 2844–2866.
Tulinsky, A., & Chien, B. M. L. (1977) *J. Am. Chem. Soc. 99*, 3647–3651.
Walker, A. F., Lo, M.-W., & Ree, M. T. (1976) *J. Am. Chem. Soc. 98*, 5552–5560.
Waterman, M. R., & Yonetani, T. (1970) *J. Biol. Chem. 245*, 5847–5852.
Yonetani, T., & Asakura, T. (1969) *J. Biol. Chem. 244*, 4580–4588.
Yoshimura, T., & Ozaki, T. (1979) *Bull. Chem. Soc. Jpn. 52*, 2268–2275.

Elastin Biosynthesis and Cross-Link Formation in Rabbit Aortic Smooth Muscle Cell Cultures†

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ABSTRACT: Rabbit aortic smooth muscle cells in culture produce insoluble elastin which can be purified by treatment with hot alkali. These cells, when maintained in the same flask for long periods of time, continue to accumulate elastin. Both desmosine and isodesmosine, cross-links unique to insoluble elastin, have also been found to increase with time in culture. The results from pulse–chase studies with radiolabeled proline and lysine confirm these observations. All the data indicate that the appearance of the desmosines in the elastin in these cell cultures is a relatively slow process, while the lysine-derived aldehydes appear quite rapidly.

The formation of insoluble elastin in in vitro cell culture systems has been the focus of several laboratories in recent years. In vivo, the disruption and/or lack of synthesis of the elastin fiber component leads to devastating diseases, such as pulmonary emphysema and, possibly, atherosclerosis. Thus, studies on the biosynthesis of the insoluble elastin fiber in cell cultures can provide valuable information to our understanding of the formation and turnover of this important connective tissue component.

Since elastic fibers are extracellular connective tissue components, the biosynthesis of the insoluble protein component has to have its origin in the intracellular protein synthetic machinery, followed by secretion from the cell and, finally, incorporation into the fiber. By examination of the end product, the insoluble elastin, one is able to learn something of a cell's ability to (1) synthesize protein, (2) hydroxylate prolyl residues, (3) transport macromolecules to the extracellular milieu, (4) assemble larger protein aggregates, and (5) form connective tissue cross-links. Studies of this type will give some insight into the mechanisms of elastin turnover.

The study of elastin synthesis in cell cultures has been limited since very few culture systems are capable of producing insoluble elastin. Thus far, the only cells which have been reported capable of producing insoluble elastin in culture are vascular smooth muscle cells (Ross, 1971; Daoud et al., 1974; Faris et al., 1976; Rucker & Tinker, 1977; Burke & Ross, 1979), human endothelial cells from umbilical cord veins (Jaffe et al., 1978), chondroblasts (Quintarelli et al., 1979), and fibroblasts from bovine ligamentum nuchae (Mecham, 1981).

As noted by several laboratories, aortic smooth muscle cells in culture are capable of synthesizing both insoluble collagen and elastin. The soluble precursors to these connective tissue proteins have also been identified in these same cell cultures (Burke et al., 1977; Scott et al., 1977; Rosenbloom & Cywinski, 1976; Uitto et al., 1976; Foster et al., 1978). Collagen has been shown to accumulate over long periods of time (Salcedo & Franzblau, 1981), and recently these cultures have been shown to accumulate glycosaminoglycans in exactly the same proportion as found in the donor rabbit aorta (Namiki et al., 1980). Few, if any, of the more recent studies have focused on the formation of the insoluble elastin in these cultures.

The studies reported here describe relatively long-term pulse–chase experiments involving the incorporation of lysine and proline into the insoluble fractions of elastin derived from the aortic smooth muscle cell layer. The increase in the total elastin content associated with the cell layer at various times in culture is also examined. The data suggest that desmosine formation is quite slow although cross-link formation (lysine-derived aldehydes) begins rapidly. Hopefully, these data will serve as the base line for future experimental protocols related to elastin fibrogenesis in cell culture systems.

Materials and Methods

Smooth Muscle Cell Cultures. Rabbit smooth muscle cells were isolated and grown from the aortic arch of weanling rabbits as described previously (Faris et al., 1976). The

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