Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 1045-1050

A Co(III) DERIVATIVE OF CONCANAVALIN A[1]

M. S. Urdea[2], D. J. Christie[3], G. R. Munske, J. A. Magnuson[4], and J. I. Legg[4]

Program in Biochemistry and Biophysics and
the Department of Chemistry
Washington State University
Pullman, WA 99164

Received October 4, 1979

SUMMARY

Co(III) has been stoichiometrically incorporated into jack bean concanavalin A. The Co(III) protein still possesses a binding site for an additional divalent transition metal ion which together with Ca(II) can induce the sugar binding ability. No H_2O_2 oxidation of Co(II) occurs with demetallized concanavalin A activated with Ca(II) and Co(II) unless Co(II) is present in a stoichiometric excess. Evidence is presented to indicate that kinetically stable Co(III) is bound to a completely different location than the thermodynamically stable Co(II) protein site.

The jack bean lectin, concanavalin A (Con A), specifically binds a variety of carbohydrates and carbohydrate-containing macromolecules (1). Until recently, it has been generally accepted that a transition metal ion [e.g., Mn(II), Ni(II), or Co(II)] and Ca(II) ion must be bound to each subunit of Con A for the saccharide-binding activity. Christie and co-workers have shown that at physiological pH Con A containing Ca(II) ions is capable of binding monosaccharides without the transition metal complement and that the addition of transition metals does not significantly alter the monosaccharide-binding capacity of the protein (2). Other investigations have indicated that Mn(II), like Ca(II), is alone capable of conferring monosaccharide-binding activity to Con A (3,4,5).

The exact role of metal ions remains unclear. The hypothesis that a transition metal must bind to its site S1 so that a calcium ion can bind to

[1]This research was supported in part by United States Public Health Service grants GM 23081 and CA 14496.

[2]Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94143.

[3]Present address: Milwaukee Blood Center, Milwaukee, WI.

[4]To whom correspondence and reprint requests should be addressed.

0006-291X/79/231045-06$01.00/0

its site S2 and induce saccharide binding activity needs further examination. To better define the role of the transition metal ion, we attempted to oxidize Co(II) at S1 to produce a substitution-inert Co(III)-Con A derivative. Unexpectedly we produced a Co(III) derivative which still required additional metal ions, such as Ca(II) and Mn(II), for saccharide binding activity. Evidence is presented which indicates that the Co(III) is neither at the S1 site nor at the S2 site, and suggests that kinetically inert Co(III) complexes can be formed in proteins at sites other than those that form thermodynamically stable Co(II) complexes.

MATERIALS AND METHODS

Con A was isolated from jack bean meal as previously described (6). An $E^{1\%}_{280}$ of 13.7 was used for the protein concentrations (7). Demetallized Con A was prepared as described by Kalb and Levitzki (8). Ca(II) contamination in the various derivatives was checked by atomic absorption. The cobalt concentrations were determined by counting of ^{57}Co (New England Nuclear) on a Beckman Biogamma II spectrometer. A specific activity of approximately 10,000 cpm per nmol of Co was used. The concentration of the Co(III) derivative was determined by biuret assay.

The Co(III) derivative was prepared as follows. To an 8.08×10^{-4} M solution of demetallized Con A in 1 M NaCl, pH 6.5, 4°C, was added 3 equivalents of $^{57}CoCl_2$. After 2 h, 1.1 equivalents of $CaCl_2$ were added and the protein was incubated for an additional 2 h. The solution was diluted with buffer to produce a solution which was 4.04×10^{-4} M Ca(II), Co(II) Con A, 0.02 M barbital, 0.5 M NaCl, 0.04 M phenol, pH 7.5 at 4°C. Phenol was added to prevent metal-catalyzed free radical damage that can occur upon Co(II) to Co(III) oxidation with H_2O_2 (9). The oxidation was initiated by the addition of enough freshly prepared H_2O_2 stock to give a 4 mM reaction solution. After an appropriate time (see Results) the reaction was quenched with catalase (1 µg/ml; Sigma) and acidified to pH 2.0 with HCl. After 30 min at 4°C, the solution was chromatographed on a G-25M Sephadex PD-10 disposable column (Pharmacia) equilibrated with 10 mM HCl. Fe(II)EDTA reduction of the Co(III) Con A derivative was carried out in 10 mM HCl with a 50-fold molar excess of the iron complex for 30 min. The Fe(II)EDTA was prepared according to Urdea and Legg (9).

The fluorescent sugar derivative, 4-methylumbelliferyl α-D-mannopyranoside (MUM), was purchased from Koch-Light Limited. The fluorescence saccharide binding assay of Christie et al. (2) was used for all Con A binding activities reported. A Perkin-Elmer MPF-3L fluorimeter was used for the binding studies.

RESULTS

Figure 1 summarizes the cobalt to Con A stoichiometries obtained at various times under a number of conditions. If Con A containing Ca(II) and Co(II) was acidified for 30 min (pH 2.0), the metal ions could be removed by

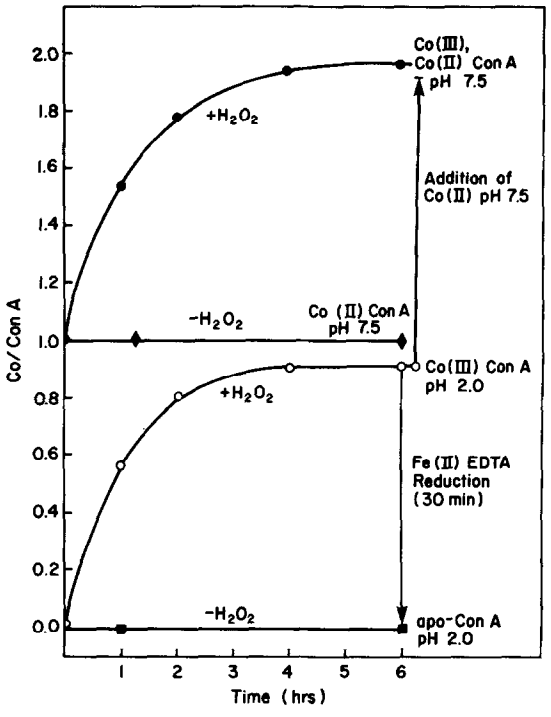

Figure 1: Cobalt stoichiometry of Con A after various treatments and chromatography on a PD-10 column. Con A with Ca(II) and excess Co(II) acidified with HCl and chromatographed at pH 2.0 (■-■). Con A with Ca(II) and excess Co(II) treated with H_2O_2, acidified, and chromatographed at pH 2.0 (o-o). Con A with Ca(II) and excess Co(II) chromatographed with pH 7.5 buffer containing 0.02 M barbital and 0.5 M NaCl (◆-◆). Con A with Ca(II) and excess Co(II) treated with H_2O_2 and chromatographed with pH 7.5 buffer (●-●). The upward arrow shows that a second cobalt can be bound to Con A containing Co(III) at pH 2.0. The downward arrow shows the demetallation step induced upon reduction.

chromatographing on a PD-10 column equilibrated with 10 mM HCl (■-■). If the protein and metal ions were not acidified, chromatography at pH 7.5 did not remove the metal ions (◆-◆). When H_2O_2 was added to oxidize the cobalt, a time-dependent increase in the amount of acid-stable cobalt was found (o-o). After approximately 4 h, a full complement of acid-stable cobalt per monomeric Con A was detected. If the protein was not acidified before chromatography at pH 7.5 following oxidation, two equivalents of cobalt were bound per 25,500-dalton subunit (●-●). If the cobalt derivative obtained after 6 h of H_2O_2 oxidation is treated with acid, chromatographed as described, brought to pH 7.5, and provided with an equivalent of Co(II), the protein binds another stoichiometric amount of cobalt (Figure 1). In addition, when Co(II) is

oxidized in the presence of Con A containing Ca(II) and Ni(II) under conditions where Co(II) and Ni(II) are slow to exchange, one acid-stable cobalt is bound to each Con A monomer (not shown).

All experiments mentioned above were carried out with a two-fold excess of Co(II) with respect to protein monomers. Only 1.03 acid-stable cobalt ions were detected when a five-fold excess was used. With a stoichiometric amount of Co(II), either in the presence or absence of Ca(II), less than 0.1 acid-stable cobalt ions could be found. Even a 50-fold excess of H_2O_2 did not give acid-stable cobalt unless excess Co(II) was present. When Co(II) is oxidized in the presence of Con A containing Ca(II) and Ni(II), acid-stable cobalt can be detected (0.96 per monomer).

The acid stable cobalt could be removed by treating the protein with a 50-fold molar excess of Fe(II)EDTA followed by column chromatography with 10 mM HCl. Over 95% of the cobalt was removed in 30 min (Figure 1). Since H_2O_2 oxidation is required for incorporation of acid-stable cobalt and Fe(II) reduction reverses the modification, it is most likely that the acid-stable cobalt is Co(III) (9).

The fluorescence assay for MUM binding to Con A, a measure of saccharide binding activity, was carried out according to Christie and co-workers (2). The Co(III) derivative, prior to the addition of other metal ions, is not active. A Scatchard plot of MUM binding to Co(III)-Con A containing Ca(II) and Ni(II) is shown in Figure 2. The results are nearly identical for the Ca(II), Co(II) protein prior to oxidation and for the Fe(II)EDTA-reduced Co(III)-Con A containing Ca(II) and Co(II) (not shown). The Co(III) derivative is also activated by Mn(II) or by Ca(II) and Mn(II).

DISCUSSION

It is evident from the data presented that Co(III) is not bound to the S1 site of Con A. The production of a Co(III) derivative of Con A containing Ca(II) and Ni(II) which binds MUM strongly supports this contention. It is

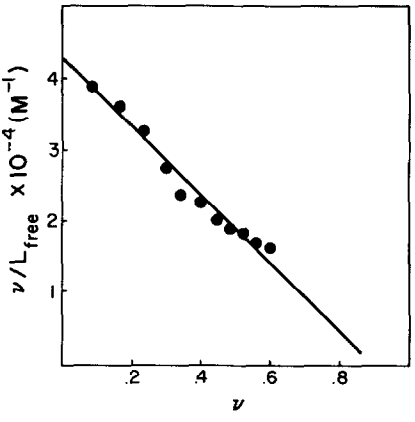

Figure 2: Scatchard plot for MUM binding to Con A containing Ca(II), Ni(II), and Co(III). L_{free} represents free MUM and ν represents fraction of carbohydrate sites occupied per 25,500-dalton subunit of Con A.

also unlikely that Co(III) occupies the Ca(II) site since the Co(III) derivative is not capable of binding MUM, although Ca(II) addition initiates saccharide-binding activity at pH 7.2, as in the native protein (2). Under the conditions investigated, Co(II) in the S1 site is not susceptible to H_2O_2 oxidation.

There are strict requirements which must be met to obtain a stable substitution-inert Co(III) complex. It is essential that six relatively strong field ligands are provided to the metal in an octahedral geometry (10). Either the ligand composition evidently is not strong enough to stabilize Co(III), or H_2O_2 may simply not reach the Co(II) in the S1 site. There is, however, another site on the protein capable of coordinating and stabilizing Co(III). It has been reported that lanthanides bind to a position other than the Ca(II) or transition metal site in Con A (12,13). Whether or not this is the Co(III) site is presently under investigation.

The utility of Co(III) as a biological probe stems from its capacity to form kinetically inert complexes that are not subject to ligand exchange (10). When Co(II) is introduced into a protein metal-binding site, it is generally assumed that Co(II) to Co(III) oxidation will occur *in situ*. This has probably occurred in a few cases (14,15). In the present study, however, a kinetically inert Co(III) complex is produced at a site other than the

thermodynamically stable Co(II) binding site. Similarly, in alkaline phosphatase, Co(II) evidently migrates from the structural to the catalytic binding site prior to H_2O_2 oxidation (14). The generality of *in situ* oxidation is therefore questionable and alternate sites of oxidation must be seriously considered in any Co(II) to Co(III) oxidation.

Acknowledgment

We would like to thank Dr. J. A. Wells for his aid in preliminary studies and for helpful discussions.

REFERENCES

1. Goldstein, I. J., and Hayes, C. E. (1978), Adv. Carbohydr. Chem. Biochem., 35, 127-340.
2. Christie, D. J., Alter, G. M., and Magnuson, J. A. (1978), Biochemistry, 17, 4425-4430.
3. Brewer, C. F., Marcus, D. M., Grollman, A. P., and Sternlicht, H. (1974), J. Biol. Chem., 249, 4614-4617.
4. Harrington, P. C., and Wilkins, R. G. (1978), Biochemistry, 17, 4245-4250.
5. Christie, D. J., Munske, G. R., and Magnuson, J. A., Biochemistry, in press.
6. Agrawal, B. B. L., and Goldstein, I. J. (1967), Biochim. Biophys. Acta, 147, 262-271.
7. Yariv, J., Kalb, A. J., and Levitzki, A. (1968), Biochim. Biophys. Acta, 165, 303-305.
8. Kalb, A. J., and Levitzki, A. (1968), Biochem. J., 109, 669-672.
9. Urdea, M. S., and Legg, J. I., Biochemistry, in press.
10. Legg, J. I. (1978), Coord. Chem. Rev., 25, 103-132.
11. Becker, J. W., Reeke, G. N., Jr., Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1975), J. Biol. Chem., 250, 1513-1524.
12. Sherry, A. D., and Cottam, G. L. (1973), Arch. Biochem. Biophys., 156, 665-572.
13. Barber, B. H., Fuhr, B., and Carver, J. P. (1975), Biochemistry, 14, 4075-4082.
14. Anderson, R. A., and Vallee, B. L. (1977), Biochemistry, 16, 4388-4342.
15. Van Wart, H. E., and Vallee, B. L. (1978), Biochemistry, 17, 3385-3394.