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# SUBSTITUTION-INERT METAL IONS AS PROBES OF BIOLOGICAL FUNCTION

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## A. INTRODUCTION

The uniquely characteristic probe capabilities of metal ions have afforded an excellent opportunity for an interdisciplinary approach to the study of metalloproteins, as well as those proteins not possessing metal ions as a co-factor [1]. Until recently the metal—enzyme systems studied have involved primarily substitution-labile metal ions (e.g., Zn(II), Mn(II), and Co(II)). Substitution-inert metal ions such as Cr(III), Co(III), and Ru(II) exhibit well established "stop-action" characteristics, and recently a number of studies have appeared which have employed the kinetic inertness of certain metal ions to study protein structure—function relationships.

Substitution-inert metal complexes have been studied extensively by inorganic chemists in order to decipher details of reaction mechanism and stereochemistry which are not accessible in the more labile systems. Certainly cobalt(III) has been exploited most fully in this respect. Substitution-inert metal ions (primarily Co(III)) have also proven valuable to biological chemists

in recent years as probes of metal ion binding sites in proteins, Table 1. Although these investigations are still in the formative stages and a considerable amount of controversy exists over the results which have been reported, the potential for generating useful information from such investigations is significant. For example, inherent in the use of substitution-inert metal ions to probe metal binding sites in proteins is the possibility of developing a method for determining protein ligating residues. Another area of biological research which is benefitting considerably from the use of substitution-inert metal ions is the study of the role of the metal ion in Mg(II) requiring enzymes. In these studies exchange-inert metal ions (primarily Cr(III)) have been substituted for Mg(II) in order to more precisely define the role of the metal ion in catalysis, Table 3. It is the intent of the review to summarize this relatively new and expanding interdisciplinary area of research as well as to point out some of the potential applications of substitution-inert metal ions. In addition problems which have arisen and precautions which need to be considered will also be discussed.

## B. GENERAL CONSIDERATIONS

### *(i) Definition of substitution-inertness*

Taube was the first to recognize that a correlation exists between *d*-orbital configuration and the reactivity of metal complexes [2]. In terms of crystal field theory, $d^3$ and strong field $d^6$ octahedral complexes are expected to exhibit the greatest kinetic stability [3]. Indeed, Cr(III) ($d^3$) and Co(III) ($d^6$) complexes are well known to be substitution-inert. For example, the rate constant for the acid hydrolysis of $Co(NH_3)_5Cl^+$ (Co(II), $d^5$) to form the pentammine aquo complex is $10^8$ $sec^{-1}$ whereas the corresponding reaction for the analogous Co(III) complex is some 13 orders of magnitude slower with $k = 6.7 \times 10^{-7}$ $sec^{-1}$ [3]. There is, of course, a continuum between inertness and lability, and under selected conditions, substitution-inert complexes can be expected to exchange certain ligands. However, the basic assumption which prevails in the experiments to be described below is that the inert complexes (protein and otherwise) under investigation are not expected to exchange ligands for the duration of the experiment.

### *(ii) Requirements for protein study*

Most applications of substitution-inert metal ions can be placed in three categories: 1) replacement of the native metal ion or metal ions in proteins by substitution-inert metal ions; 2) binding of substitution-inert metal ions to non-native metal ion binding sites in proteins; 3) complexation of substitution-inert metal ions by substrates. In general, proteins are very sensitive to environmental changes and are stable over relatively small ranges of temperature and pH. Such stringent requirements might be expected to pose serious

problems since, as expected, many reactions involving substitution-inert metal ions often require extreme conditions of temperature and pH. What is needed for the protein studies is a metal ion in a state where it can be easily inserted into the protein matrix and then altered to a substitution-inert state under mild conditions. Perusal of the periodic table reveals several candidates, prime among which is cobalt. In the +2 oxidation state cobalt is very substitution-labile. When introduced into a good chelating environment or in the presence of moderately strong field ligands such as amines or amino acids, Co(II) often will be oxidized spontaneously to substitution-inert Co(III) in the presence of atmospheric oxygen. Chromium is in a similar situation but labile Cr(II) is much more sensitive to oxidation than Co(II) and normally must be handled under rigorously anaerobic conditions. To date Co(III) and Cr(III) have been used almost exclusively in the study of protein structure and function, but it is anticipated that other substitution-inert metal ions will eventually be found useful for certain studies. For example, in the presence of $\pi$-acid ligands such as aromatic amines, Ru(II) forms complexes which are both kinetically stable to substitution and oxidation [4]. Since many metal ion sites in metalloenzymes involve one or more histidines as ligands, ligated through the imidazole side chain, it might be expected that Ru(II) could be incorporated into such sites. However, it has been observed that imidazole loses some of its $\pi$-acidity due to a relatively large $\sigma$-base contribution and thus falls somewhere between ammonia and pyridine in its stabilization of Ru(II) [5], and thus Ru(II) complexes involving ligation of the imidazole side chain of histidine might be expected to be somewhat labile.

### C. APPLICATIONS *

Although it is not the purpose of this review to discuss model systems except where they may bear directly on some aspect of the protein work to be discussed, it is well worth mentioning a series of studies on the Co(III) promoted hydrolysis of peptides and esters which culminated in the elegant study of Buckingham, Sargeson and coworkers [6]. By using $^{18}O$ labeled water they were able to establish both an inter- and intramolecular mechanism for metal ion promoted hydrolysis of glycine amides. The intramolecular pathway, where the metal ion functions as a localized source of base, was shown to be $10^7$ (possibly more than $10^{11}$) times faster than hydrolysis of uncoordinated glycine amide. The rate compares with an enhancement of $10^4$ over the uncoordinated amide for the intermolecular pathway where the amide carbonyl

* The review will focus on studies where the primary emphasis has been on the utilization of substitution-inertness to probe biological function. Thus, investigations in which cobalt has been substituted into heme proteins will not be discussed, since these studies are not for the most part dependent on the property of substitution inertness. It should also be noted that substitution-inert metals (e.g., Pt) have been used as heavy metal labels in proteins for crystallographic studies.

is directly bound to the metal ion. The authors concluded that the more efficient intramolecular pathway might be operative in the hydrolytic enzymes such as carboxypeptidase A and carbonic anhydrase. Carbonyl activation by the metal ion of the substrate to be hydrolyzed has been postulated for metallohydrolysases.

The application of substitution-inert metal ions to the study of biological function can be divided conveniently into four topics which are discussed below. The first of these deals with the replacement of native metal ions in metalloproteins and enzymes by substitution-inert metal ions and the resulting alteration in enzymatic behavior. Most of this work has been concerned with zinc metalloenzymes. The second topic discussed is an extension of the first, but because of its potential importance is given a separate heading. This section addresses the use of substitution-inert metal ions to determine ligating residues in proteins. Although a number of laboratories are currently actively involved in such studies, only two reports have been published to date. Therefore the discussion in this section deals with the potential of the technique as well as reviewing the published work. The third section discusses the rather extensive and proliferating literature concerned with the use of substitution-inert metal ions, in particular Cr(III), to study Mg(II) requiring enzymes. The fourth topic deals with the specific binding of substitution-inert metal ions to enzyme residues, primarily to determine whether such residues are involved in the catalytic mechanism. Other applications which might be of interest are summarized in the last section.

*(i) Replacement of native metal ions in metalloproteins with substitution-inert metal ions*

Kornfield [7] and Aisen and co-workers [8] were the first to report replacement of a native metal ion in a nonheme protein with a substitution-inert metal ion. In the former study Cu(II) and Cr(III) were introduced into apotransferrin (a plasma glycoprotein involved in Fe(III) transport to immature red blood cells) and were thought to bind to the two Fe(III) sites in the protein. As expected the Cr(III)-substituted enzyme exhibited biological and chemical properties more like those of the Fe(III) protein than did the Cu(II)-substituted protein. In the latter study the two irons in transferrin were replaced with Co(III) as well as Cr(III). Hydrogen peroxide was used to oxidize Co(II) in the cobalt substituted protein. Residual paramagnetism was detected in the sample and attributed to the presence of about 12% Co(II). However, the failure to detect an EPR signal down to 77 K is not evidence for the absence of Co(II) as claimed by the authors. Detection of a high-spin Co(II) signal normally requires liquid helium temperatures as has been demonstrated for a series Co(II) substituted metalloenzymes [9]. In addition the electronic absorption spectrum obtained (blanked against apotransferrin), a broad band at 450 nm, is not consistent with any known Co(III) complex. On the other hand, the pale blue Cr(III) protein exhibits a characteristic Cr(III) spectrum

TABLE 1

Protein bound Co(III) and Cr(III)

| | Protein | Metal | Ref. |
|---|---|---|---|
| Native metal ion binding sites in metalloproteins | Transferrin | Co, Cr | 7, 8 |
| | Carboxypeptidase A [a] | Co | 11, 15 |
| | Carbonic anhydrase B [a] | Co | 15,16 |
| | Alkaline phosphatase | Co | 32 |
| | Concanavalin A | Co | 15 |
| | Cytochrome *c* | Co | 78 |
| Other metal binding sites in proteins | Cytochrome *c* [a] | Cr | 34, 35 |
| | Aspartokinase-homoserine dehydrogenase | Co | 36, 37, 38, 51, 52 |
| | Yeast enolase [a] | Co | 42 |
| | Arsanilazotyr-248 carboxypeptidase A | Co | 71, 74, 75 |

[a] Proteolysis of substitution-inert metal protein complex attempted.

consisting of two weak, broad maxima at 615 and 440 nm. Such a spectrum would result from a ligand field somewhat weaker than six waters [10], (e.g., six acetates). Analysis of the EPR spectrum of the Cr(III) analogue suggested that the two metal binding sites in transferrin are not identical [8]. However, neither of these investigations made use of the substitution-inertness of the metal ions involved.

Studies in which Co(III) or Cr(III) are thought to be bound to protein metal ion binding sites (native or otherwise) are listed in Table 1. Of particular interest are the recent studies which have appeared, concerned with the oxidation of Co(II) in Co(II)-substituted zinc metalloenzymes. The appropriateness of using cobalt as a substitution-inert metal ion probe was discussed in the previous section. In investigations involving zinc metalloenzymes, cobalt has an additional advantage in that it has been sucessfully substituted as Co(II) in a series of zinc metalloenzymes [1]. Furthermore, of the divalent metal ions, Co(II) appears to be the most comparable in chemical behavior with the native Zn(II) ion. Whereas many divalent metal ions inactivate or dramatically alter enzyme activity when substituted for Zn(II), Co(II) often produces a metalloenzyme of comparable activity to the native enzyme. Thus, not only are procedures already available for Zn(II) replacement by Co(II) but it would appear, at least in the +2 oxidation state, that no major alteration of enzyme conformation (stereochemistry) occurs on replacement, a situation which is desirable when incorporating enzyme probes.

Upon replacing Zn(II) with a substitution-inert metal ion, the following result might be anticipated. If the Zn(II) is in the active site and the substrate

binds directly to the metal ion during catalysis, then replacement of the Zn(II) by a substitution-inert metal ion would lead to inactivation since the substrate would be expected to form a kinetically stable bond to the metal ion. Thus far, three Co(II)-substituted zinc metalloenzymes have been submitted to oxidation; carboxypeptidase A, carbonic anhydrase B, and alkaline phosphatase (Table 1). In the first reported of these studies, Storm and co-workers [11] used a dilute hydrogen peroxide solution to achieve oxidation of Co(II)-carboxypeptidase A (CPD) * at pH = 7.5. At higher peroxide concentrations oxidation of protein residues (methionine, tryptophan, cysteine) is likely. As a control they submitted Zn(II)-CPD to the same conditions used for the Co(II) enzyme and found no alteration of peptidase or esterase activity. Amino acid analysis of the two hydrogen peroxide treated enzymes suggested that both methionine and cysteine were unaltered by peroxide treatment, but under the acid hydrolysis conditions employed it was not possible to determine whether any tryptophan had been oxidized. In addition it should be noted that if methionine sulfoxide had been produced during peroxide treatment, it would have been reduced back to methionine under the acid hydrolysis conditions employed for the amino acid analysis [12]. Cobalt analysis showed the modified CPD to have a cobalt : protein molar ratio of 0.95—1.20.

Dialysis of the Co(III) enzyme against the chelating agent 1,10-phenanthroline did not remove the cobalt [11]. This chelating agent is very effective in removing substitution-labile Zn(II) from CPD [13]. Dialysis of $^{57}$Co(III)-CPD against Co(II) restored peptidase activity (absent in the Co(III) enzyme, vide infra) with a concomitant release of radioactive cobalt. Electron transfer between Co(II) and Co(III) often occurs under mild conditions leading to the labilization of Co(III) complexes [14]. The electronic absorption spectrum obtained (maximum at 503 nm with $\epsilon$ = 500) is consistent with Co(III) although the molar absorptivity is somewhat high. These observations support the presence of Co(III) in the peroxide treated enzyme. However, the definitive liquid helium EPR experiment which would conclusively demonstrate the absence of Co(II) was not reported.

Kang et al. found that Co(III)-CPD lost peptidase but retained esterase activity and concluded that the substrate must bind directly to the metal ion during peptide but not ester hydrolysis [11]. Therefore, it is unlikely that ligand—metal bond breaking is involved in ester hydrolysis. In addition they found that a peptide substrate for the Co(II) enzyme competitively inhibited the esterase activity of the Co(III) derivative indicating that the lack of peptidase activity was not due to inability of the enzyme to bind peptide sub-

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* Carboxypeptidase A (CPD or CPA) is a Zn(II) metalloenzyme containing 307 amino acids with a molecular weight of 34,600. As the name implies it cleaves amino acids from the carboxyl terminal end of peptides. It also hydrolyzes esters. The crystal structure shows that the zinc is at the active site bound to two histidines and a glutamic acid as well as a water molecule in the crystalline state (see refs. 20, 21, 60 and 70).

strates. Recent studies by Storm and coworkers [11b] indicate that the Co(II) replacement process in CPD is more complex than first envisaged. They found that if the Co(II) enzyme is prepared by mass action of an excess of Co(II) dialyzed against the Zn(II) enzyme, $H_2O_2$ oxidation yields the product previously characterized. On the other hand if the Co(II) enzyme is prepared by first forming the apoenzyme followed by addition of Co(II), $H_2O_2$ oxidation yields a product which lacks both peptidase and esterase activities.

Very recently Van Wart and Vallee [15] reported the preparation of a totally inactive Co(III)-carboxypeptidase A by employing an active-site directed oxidizing agent, *m*-chloroperbenzoate, in the presence of the radical scavenger phenol. Affinity chromatography yielded a homogeneous product with 0.95 g-atom Co and 0.01 g-atom Zn per mole enzyme. The product has the same molecular weight and amino acid composition as the native enzyme and was devoid of both peptidase and esterase activity. Importantly no EPR signal was present at 5 K indicating the absence of Co(II). Vallee and coworkers also attempted to prepare Co(III)-CPD employing the method of Storm and coworkers [11]. Gel filtration of the oxidation products resulted in a heterogeneous mixture of proteins containing non-stoichiometric quantities of cobalt and exhibiting variably reduced activities. In addition EPR spectra revealed that a substantial amount of Co(II) was still present. It should be noted that the method employed for preparation of Co(II) substituted CPD referred to was different from that of Storm and coworkers.

These recent studies suggest that a rather complex situation exists for the oxidation of Co(II) substituted CPD and that no definite conclusions can be reached at present. It would appear that the method of preparation of Co(II)-CPD can influence the products obtained on oxidation. In addition the oxidizing agent employed is important. Finally, in the case where *m*-chloroperbenzoate is used, it is conceivable that the reduced active site reagent remains ligated to the Co(II) blocking the active site pocket and inhibiting the enzyme. This observation is supported by the fact that the potent inhibitor β-phenylpropionate prevents the oxidation of Co(II)-CPD when *m*-chloroperbenzoate is added [15] presumably by preventing contact between the oxidizing agent and Co(II).

In a similar study Shinar and Navon [16] carried out the oxidation of Co(II)-substituted carbonic anhydrase (CA) *. They found an electronic absorption spectrum (a broad band at ca. 520 nm with $\epsilon$ ca. 370) consistent with Co(III) but gave little other evidence for the presence of Co(III). They also noted that if the $H_2O_2$ concentration exceeded a certain excess, denatura-

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* Carbonic anhydrase is a Zn(II) metalloenzyme with about 260 amino acids and a molecular weight of around 30,000 (there are different forms). One of its physiological roles is the fast hydration of $CO_2$ in various tissues and the dehydration of $HCO_3^-$ in the lungs. Crystal structures of two forms have been reported and show the zinc to be at the active site bound to three histidines and one water molecule in the crystalline state (see refs. 23 and 24).

tion of the enzyme took place. The native zinc enzyme was unaffected by peroxide treatment, but Co(II)-CA, on oxidation, lost all activity towards hydration of carbon dioxide and hydrolysis of the synthetic substrate, *p*-nitrophenyl acetate. They postulated that the substrate must bind directly to the metal ion during catalysis, the same conclusion reached by Storm and co-workers in their study of carboxypeptidase A. However, the information supplied in this brief report is insufficient to come to any definitive conclusion.

These two studies on the oxidation of Co(II) to Co(III) in bovine carboxypeptidase A and carbonic anhydrase B, although of potential significance, have failed to take into consideration important implications arising from the strong preference of Co(III) for an octahedral environment. A structural feature inherent to Co(III) is that all known Co(III) complexes which are commonly encountered in aqueous solutions are six coordinate and almost always octahedral *. Small distortions from octahedral geometry have been indicated from absorption spectral studies [18], and crystallographic studies show that some chelate rings impose distortions of from 4° to 6° from the idealized 90° bond angles of an octahedron [19]. However, in aqueous solution Co(III) complexes have six coordinated ligands which are usually in an octahedral array.

It has been shown that in both the crystalline state [20] and in solution [21] the active site metal ion in carboxypeptidase is in a distorted (and most likely four coordinate) environment. Although there is some question as to

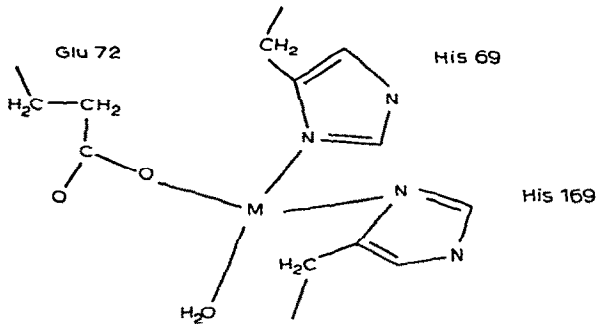
TABLE 2

Crystal structure data for various Zn(II) metalloenzymes

| Enzyme | Metal Ion(s) | Ligating Residues | Ref. |
|---|---|---|---|
| Alcohol dehydrogenase (horse liver) | Zn | Cys 46, 174<br>His 67 | 26 |
| | Zn [a] | Cys 97, 100, 103, 111 | |
| Carboxypeptidase A | Zn | His 69, 196<br>Glu 72 | 20 |
| Carbonic anhydrase B | Zn | His 94, 96, 119 | 23 |
| Carbonic anhydrase C | Zn | His 93, 95, 117 | 24 |
| Superoxide dismutase | Zn | His 61, 69, 78<br>Asp 81 | 27 |
| | Cu | His 44, 46, 61, 118 | |
| Thermolysin | Zn | His 142, 146<br>Glu 166 | 28 |

[a] Structural site Zn's.

* Several square-pyramidal complexes involving macrocycles have been reported, but these are generally unstable, particularly in polar media [17].



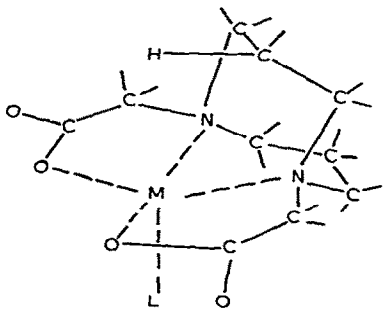




Fig. 1. Metal ion site in carboxypeptidase A and dacoda analogue.

the number of ligands associated with the active site metal ion in carbonic anhydrase [22], the crystal structure of human carbonic anhydrase B and C [23,24] and absorption spectral studies of the active Co(II) substituted enzyme [25], also support a distorted tetrahedral or possibly five-coordinate geometry for the active alkaline form. In fact, it would appear that for most of the Zn(II) metalloenzymes whose structures have been determined by X-ray crystallography, the metal ion is in a distorted environment with a coordination number less than six (Table 2).

In order to probe the possible importance of an irregular metal ion environment to catalysis in carboxypeptidase A (CPD), a series of chelates having the same type of ligating atoms (two nitrogens and two or three oxygens) and the same approximate geometry as the native Zn(II) in CPD were synthesized and characterized, (Fig. 1) [29]. Significantly, a study by Gray and coworkers [30] has shown that in Ni(II)-substituted CPD, an active derivative, the inhibitor β-phenylpropionate forms a complex with the enzyme whose spectral properties are strikingly similar to those of one of the model Ni(II) chelates, Ni(dacoda)($H_2O$) (dacoda = 1,5-diazacyclooctane-*N*,*N'*-diacetate)

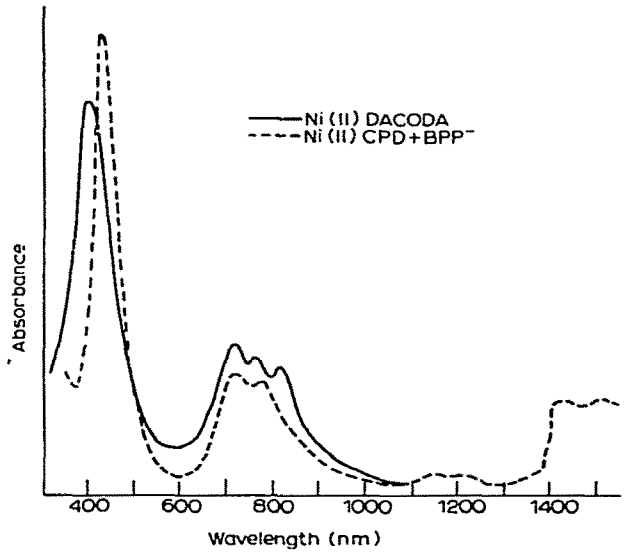



Fig. 2. Absorption spectrum of Ni(II) substituted carboxypeptidase A plus β-phenylpropionate ($BPP^-$) and Ni(II)-dacoda in aqueous solutions.

(Fig. 2). In a recently reported spectral study on anion binding to Co(II)-substituted carbonic anhydrase [31], an absorption spectrum was obtained for acetate binding to the Co(II) enzyme which is very close to that observed for Co(dacoda)($H_2O$) [29] with respect to both the positions of the absorption maxima and the relative intensities of the bands. The authors postulated from an analysis of the spectrum that the Co(II)-enzyme-acetate complex was five-coordinate. Of particular importance to the oxidation of protein bound Co(II) is the observation that these model chelates prevent a metal ion from attaining octahedral coordination in an aqueous environment and that it has not yet been possible to oxidize Co(II) complexes of these ligands, even though analogous Co(II) complexes of ligands which do not prevent a metal ion from achieving six-coordination readily oxidize to the corresponding Co(III) complex [29]. The strong preference of Co(III) for a six-coordinate octahedral geometry apparently inhibits the oxidation of the five-coordinate Co(II) complex.

In the light of these observations it is of interest to reconsider the oxidation of Co(II) in carboxypeptidase A and carbonic anhydrase B. Working with the reasonable hypothesis that if oxidation of the active site Co(II) in these Co(II) derivatives occurs, the resulting Co(III) environment must be octahedral, two distinct possibilities exist:

(i) The Co(III) is in an octahedral environment in essentially the same site as was occupied by the Co(II), most likely retaining the original protein residues and adding sufficient solvent molecules or possibly protein residues to attain a six-coordinate geometry.

(ii) The Co(III) is in an octahedral environment but is no longer in the original site.

If case (i) obtains, it is likely that a conformational change in the enzyme would have to occur to accommodate a change of the active site complex from distorted tetrahedral to octahedral. Any alteration in the observed behaviour of the Co(III) enzyme could be attributed to the change in metal ion environment, and/or to repositioning of other catalytically important groups. In either case mechanistic deductions based on comparing the Co(III) and native enzyme behavior would be suspect.

If case (ii) obtains, the enzyme should inactivate due to the removal of the metal ion from the active site, provided that replacement of the Co(II) by adventitious metals (e.g., Zn(II)) does not occur. If replacement with Zn(II) were to occur, and the activity of the "Co(III) enzyme" changed, such behavior could be attributed to the formation of a Co(III) complex with a residue important to catalysis. Such a possibility is discussed in Sec. C-(iv).

It should be noted that oxidations of Co(II)-CPD are carried out in the presence of excess Co(II) which is needed to stabilize the enzyme at the concentrations being investigated ($10^{-4}$ M) [11a]. Thus, it is possible that a Co(III) bound at a site other than the native site could be produced and a situation similar to that described above could occur.

A third possibility exists, although the constraints imposed by the model studies dictate against this alternative:

(iii) The Co(III) maintains the same ligating atoms and geometry as had the Co(II).

The latter possibility is more intriguing from a chelate chemist's point of view, since it suggests that if a sufficiently complex ligand is constructed, it may be possible to force a metal ion with a strong geometrical preference into a nonconventional environment. Although chemists have been particularly adept at imposing unusual geometries on the more geometrically flexible metal ions, Co(III) still remains a challenge. If case (iii) is realized, the Co(III) would then be in a distorted tetrahedral or possibly five-coordinate environment. The electronic configuration for a tetrahedral environment would be either $e^4t_2^2$ (strong field) or $e^3t_2^3$ (weak field). In either case the Co(III) would be paramagnetic (2 unpaired electrons in the former and 4 unpaired electrons in the latter) and easily detected. It is clear that arguments based on strong field Co(III) model octahedral systems would not apply, since, for example, it cannot be concluded that such a Co(III) complex would be inert.

A particularly relevant study to the general consideration of oxidation of Co(II) substituted metalloproteins is that of Anderson and Vallee [32] on *E. coli* alkaline phosphatase. Alkaline phosphatase is a highly complex metalloprotein for which 3 pairs of metal ion binding sites have been strongly suggested by Co(II) substitution studies and metal analysis of the native enzyme [33]. The activity and spectral properties of the Co(II) substituted enzyme are altered significantly by magnesium which appears to occupy one

pair of sites. Spectral titrations of the apoenzyme with Co(II) show the presence of one pair of octahedral Zn(II) binding sites. Characteristically weak absorption spectra and ESR and MCD spectra resembling spectra obtained for octahedral Co(II) model complexes are observed. When the titrations are repeated after pre-equilibrating the enzyme with Mg(II) (Mg(II) does not bind to the zinc sites) spectra characteristic of distorted five- or four-coordinate Co(II) are generated. It is clear from these studies that Mg(II) is regulating metal ion distribution and/or metal ion environment, possibly through conformational changes of the protein. Oxidation of various forms of the Co(II)-substituted alkalinephosphatase (the 2 Co(II) and 4 Co(II) enzyme as well as zinc and cobalt hybrids) results in the oxidation of only one pair of cobalts and does not affect any of the amino acid residues. Thus-far all oxidation products obtained are inactive, have absorption spectra characteristic of Co(III) in an octahedral nitrogen-oxygen environment ($\lambda_{max}$ = 530 nm with $\epsilon$ = 125 per Co(III)) and have no ESR signal or exhibit a 50% reduction in signal in the case of the product obtained from oxidation of the 4 Co substituted enzyme. Important to the discussion in this section is that only two Co(II)'s oxidize which is consistent with the presence of two octahedral Zn binding sites or two Zn sites whose geometry is regulated by Mg(II).

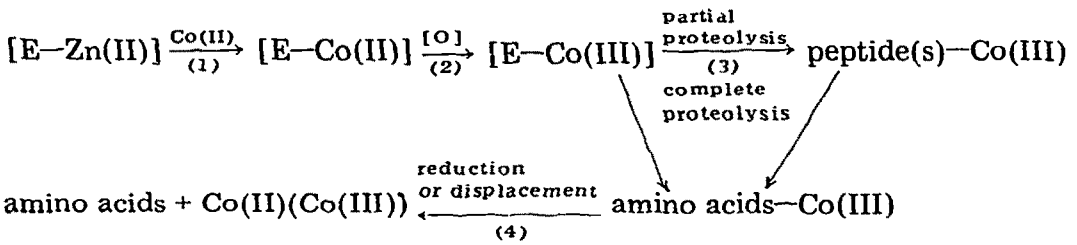
What happens on oxidation of Co(II) in nonoctahedral sites in metalloproteins which are not geometrically flexible is very much an open question. Most important to these studies would be an experiment to determine whether Co(III) was still bound to the residues shown to be coordinated to the native metal ion (Zn(II)) in the enzyme. The potential for such an experiment exists and is based on the presence of a substitution-inert metal ion at a protein binding site as described in the following section.

*(ii) Determination of ligating residues in metalloproteins with substitution-inert metal ions*

The Cr(II) to substitution-inert Cr(III) oxidation has been used to probe the route of electron transfer in cytochrome *c* [34,35]. Introduction of Cr(II) to a solution of ferricytochrome *c* produces ferrocytochrome *c* with the formation of a one-to-one Cr(III) protein complex. The basic premise was that Cr(III) would label the site of electron transfer. Due to its substitution inertness it would not be expected to migrate after electron transfer. Kowalsky [34] subjected the Cr(III) labeled cytochrome *c* complex to pepsin digestion with the expectation that the label would remain at the original site. A crude fraction containing Cr(III) was isolated but no further breakdown of this fragment was attempted. Fleischer and co-workers [35] subsequently improved the degradation of the labeled protein by using a sequence of peptidase digestions (trypsin and amino peptidase M). Two Cr(III) cross-linked fragments were isolated and identified by amino acid analysis. From an examination of a model of cytochrome *c* they were able to locate two residues (a tyrosine and an asparagine) capable of ligating the same metal ion.

Takahashi and co-workers [36,37] have used peroxide oxidation of Co(II), which will substitute for Mg(II) for aspartokinase activity, to label the ATP binding site in aspartokinase-homoserine dehydrogenase (see Sect. C(iii)). It is thought that the Mg(II) cofactor may function to bridge the enzyme with ATP. They first employed limited chymotrypsin [36] and later trypsin digestion [38] to isolate a small ternary complex involving Co(III), ATP, and a small peptide consisting of 17—18 amino acids.

These experiments suggest a potentially powerful approach for establishing ligating residues in metalloenzymes where Co(II) can replace the native metal ion (e.g. Zn(II)) as outlined below:




The procedure involves (1) substitution of Co(II) for the native metal ion(s) (Zn(II)), (2) oxidation of Co(II) to Co(III), (3) proteolysis to eventually remove all but the coordinated amino acids, and (4) removal of the cobalt, followed by determination of the amino acids present. The critical, and as yet, poorly understood steps are (2) and (3). The preliminary studies and problems associated with step (2) were discussed in Sect. C(i). In particular it was noted that due to the non-octahedral sites occupied by many metal ions in metalloproteins, a substituted Co(II) might be expected to relocate on oxidation in order to attain an octahedral geometry. In the studies employing Cr(III) in an attempt to label the electron transfer site in cytochrome *c*, it was pointed out that the possibility of rearrangements and ligand substitutions would make the determination of the original Cr(III) protein ligands ambiguous. In this light the need for model studies was stressed [34,35]. Thus, it is critical to distinguish migration during oxidation from migration during proteolytic digestion (step (3)) of the Co(III) protein.

In order to help answer these questions the synthesis and proteolysis of Co(III)-peptide complexes whose structural features bear some similarity to those expected for the Co(III)-protein complexes has been undertaken [39]. In order to select an appropriate peptide the structural data available for Zn(II) metalloenzymes were examined. The metal ion binding sites in metalloenzymes whose completed or nearly completed crystal structures have been reported (Table 2) possess several features in common: they have one or more histidines coordinated to the metal ion(s) and they have at least two residues which are close in the peptide chain, from 1 to 3 residues apart. These two properties suggested construction of a polypeptide analogue with histidines in which at least two potentially ligating residues are in a similar configura-

tion to those found in the enzymes. A detailed consideration of various sequences with molecular models led to the selection of the decapeptide GlyHisGlyGlyHisGlyGlyHisGlyGly. This peptide was produced using a modification of the Merrifield solid phase synthesis [40] and its ligating properties are currently under investigation [39].

Recently Storm and co-workers [11,41] and Rose and Westhead [42] have undertaken the proteolytic digestion of a number of Co(III)-protein complexes with mixed degrees of success. These researchers have also recognized the need for model studies to better define the problems associated with the proposed method.

Finally it should be noted that formation of substitution-inert metal ion complexes in proteins (metallo and otherwise) could prove quite valuable in both sequencing and determining conformations of proteins in solution. In effect, the substitution-inert metal ion functions as a cross-linking reagent. Thus, partial proteolysis in the presence and absence of a bound substitution-inert metal ion might be expected to give different fragments depending on where the protein is held together by the metal ion.

*(iii) Probing the function of Mg(II) in Mg(II)-requiring enzymes with substitution-inert metal ions*

*Enzyme Studies.* A significant number of the currently purified enzymes require Mg(II) and a nucleotide (usually ATP) as a substrate. The metal ion and nucleotide most likely function as a complex. A tremendous amount of research has gone into determining how ATP binds to enzymes and what role, if any, Mg(II) plays in nucleotide binding. Whether the magnesium functions simply as a counter ion, is involved in bringing about a specific geometry of nucleotide necessary to effect binding, and/or functions to bridge substrate and enzyme, is still for the most part an open question. Indeed, it is most likely that the metal ion role will vary from one class of enzyme to another as well as within a class of enzymes. Unfortunately, due to the substitution-lability of Mg(II) it is difficult to study the state of coordination of the nucleotide substrate in the active complex. Replacement of Mg(II) with Cr(III) and Co(III) to produce substitution-inert analogues of the magnesium—substrate complexes has permitted investigators to begin to probe the possible role of the metal ion in substrate binding as well as to begin to decipher the geometry of the nucleotide binding sites. As expected these substitution-inert analogues function as inhibitors in most cases. This application of substitution-inert metal ions to biological function has received rapidly increasing attention over the last several years as summarized in Table 3.

Before reviewing some of these applications it should be noted that synthesis and characterization of Cr(III) and Co(III) nucleotide complexes has been critical to the interpretation of much of the data which has appeared. Unfortunately, with the possible exception of the studies reported by Cleland, (vide infra) this is a problem which has not yet been adequately solved. This

TABLE 3

Substitution of Cr(III) and Co(III) to probe metal ion function in Mg(II) requiring enzymes

| Enzyme | Source | Metal ion substituted | Ref. |
|---|---|---|---|
| Acetate kinase | *E. coli* | Cr(III) | 46, 83 |
| Adenosine triphosphatase | Rat liver and beef heart mitochondria | Cr(III) | 53 |
| Aspartokinase-homoserine dehydrogenase | *E. coli* | Co(III) | 36, 37, 38, 51, 52 |
| Creatine kinase | Rabbit muscle | Cr(III) | 45, 46 |
| Glycerokinase | *Candida mycoderma* | Cr(III) | 46, 84 |
| Hexokinase | Yeast | Cr(III) | 44, 46 |
| Inorganic pyrophosphatase | Yeast | Cr(III) | 55 |
| Myosin | Rabbit muscle | Co(III) | 58 |
| Phosphoglycerate kinase | Yeast | Cr(III) | 46, 83 |
| Phosphorylase *b* | Rabbit muscle | Co(III) | 57 |
| Pyruvate carboxylase | Rat liver | Cr(III) | 54 |
| Pyruvate kinase | Rabbit muscle | Cr(III) | 46, 47, 48 |

problem will be addressed separately at the end of this section.

Because of the uncertainty in the identity of the substitution-inert nucleotide complexes used in these studies, it would be of little value to discuss in any detail many of the conclusions reached. However, some of the conclusions are dependent only upon having a nucleotide complex and not on its detailed structure, and these results will be reviewed. Detailed knowledge of the stereochemistry and chemical behavior of the nucleotide complexes used in the enzyme studies could confirm (or refute) the interpretation of the data obtained, and some of these results will also be summarized in order to point out the (potential) probe capabilities of substitution-inert nucleotide complexes.

Cleland and co-workers [43,44] have been instrumental in introducing the technique through the use of Cr(III)-nucleotide complexes which they have employed to study metal ion—nucleotide—enzyme interactions in a variety of kinases (enzymes which transfer a phosphoryl group from nucleotides to a variety of substrates), Table 3. Several other research groups have employed Cleland's complexes or used his reported synthesis to obtain the analogues used for their studies. Schimerlik and Cleland found that CrATP [55] *, a competitive inhibitor of creatine kinase, had a ten-fold greater affinity for the enzyme over MgATP. They postulated that MgATP exists as an equili-

* The notation employed here is that used in the biochemical literature. CrATP means that a complex exists between Cr(III) and ATP. If ATP is coordinated as a tridentate ligand, as presumed in this case, the remaining coordination sites are occupied by water. Occasionally coordinated waters are indicated explicitly as in $Cr(NH_3)_4(H_2O)ATP$. Clearly, in this case, ATP coordinated as a monodentate is implied.

brium distribution of doubly ($\beta$, $\gamma$, Fig. 3) * and triply ($\alpha$, $\beta$, $\gamma$) coordinated polyphosphate, and concluded that triply coordinated ATP is probably the actual substrate since ATP is triply coordinated in the Cr(III) complex. This assumption is based on the correct characterization of CrATP (vide infra). No mention was made of the possible effect on binding of the charge difference expected between CrATP and MgATP although later studies have recognized that difference in charge between various metal-nucleotide complexes (or analogues) could account for differences in binding [46] and reactivity [47].

In a later study Janson and Cleland [46] examined the inhibition of a series of kinases (Table 3) as a function of base (adenine, cytosine, guanine, uracil, hypoxanthine), Fig. 3, making up the nucleotide (both di- and tripolyphosphates) coordinated to Cr(III). They found that with most of the kinases the analogues were effective inhibitors and bound as well or better than the Mg(II)-nucleotides. They observed a correlation between the analogue and the corresponding Mg(II)-nucleotide. This suggests that the recognition site on the kinases is geared in part to the base component of the nucleotide. This was not the only factor involved in substrate binding, since Cr(III)-D-ribose-5′-tripolyphosphate and Cr(III)-di- and tripolyphosphates (no base present) also functioned as effective inhibitors. However, there are charge differences among the various analogues which could affect the mode of binding.

CrATP forms a kinetically stable complex with yeast hexokinase when incubated with the enzyme in the presence of the substrate glucose [44]. Acid denaturation of the resulting enzyme complex released 25% of the sugar as free glucose and 75% as a glucose-6-phosphate (product of the phosphoryl transfer from ATP) complex of CrADP. Also it was observed that prolonged incubation with glucose and other sugars released the CrADP-sugar-6-phosphate complex, so that CrATP is a very poor substrate for the enzyme. These studies suggest an intimate role for Mg(II) in phosphoryl transfer. The observations would not have been possible without the use of a substitution-inert metal ion. However, it is noteworthy that some lability was clearly necessary for the observed residual activity. Similar studies with Co(III)-ATP might not have been possible due to the greater substitution-inertness of this metal ion.

It was also observed that bidentate $Cr(NH_3)_4ATP$ ($\beta$, $\gamma$ coordinated, Fig. 3) and monodentate CrADP ($\beta$-coordinated) but not bidentate CrADP were good inhibitors [44]. This suggested that the actual substrates are $\beta$-$\gamma$-bidentate MgATP and $\beta$-monodentate MgADP. The conclusion is consistent with the slow turnover of CrATP since breaking of the inert $\gamma$-phosphate bond would be involved. It might be expected, then, that if $\alpha$,$\beta$-bidentate CrATP were employed, a rapid initial rate would be observed since phosphoryl transfer would not involve metal—ligand bond breaking. Again, these observations

* In Cleland's studies coordination through only the polyphosphate oxygens is thought to take place. Coordination sites on the sugar and nitrogen base, Fig. 3, have been considered by others (vide infra).

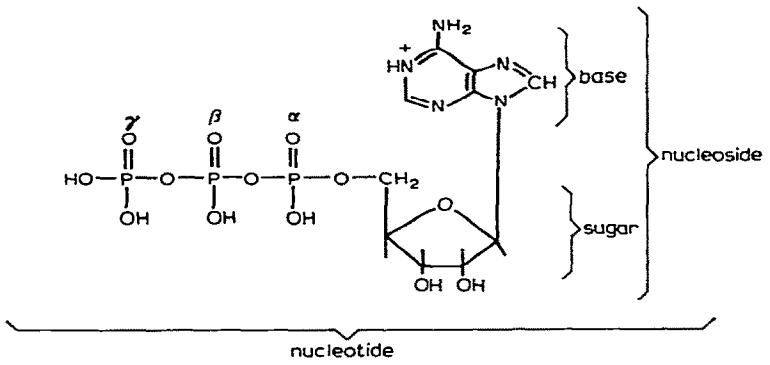



Fig. 3. Nucleotide nomenclature as exemplified for ATP. ATP shown in fully protonated form ($pK_1$ = ?).

are based on correct structural assignments of the Cr(III) analogues.

Mildvan and co-workers [47,48] employed both the substitution-inertness and paramagnetism of Cr(III) as well as the paramagnetism of Mn(II) to help establish the role of Mg(II) in catalysis by rabbit muscle pyruvate kinase. The enzyme reversibly transfers the phosphoryl group of phosphoenolpyruvate to ADP and also catalyzes the enolization of pyruvate. The enzyme is a tetramer and requires two $Mg^{2+}$ per subunit, one directly bound to the enzyme and the other bound only to the substrate. It had been previously demonstrated that Mn(II) substitutes for Mg(II) to give an enzyme with 52% of the original activity [49]. They found that CrATP (prepared by the method of dePamphilis and Cleland [43]) replaced ATP in promoting enolization only in the presence of Mg(II) or Mn(II). From NMR studies Reuben and Cohn [50] had estimated that in the Mn-enzyme complex there are only three fast exchanging waters, the remaining three ligands arising from the protein. Upon addition of CrATP Mildvan and co-workers observed a de-enhancement of the paramagnetic effect of the enzyme-bound Mn(II) on the water relaxation rate indicating the formation of an enzyme-Mn(II)-ATP-Cr(III) complex. On addition of pyruvate no fast exchanging waters were observed indicating that solvent accesss to the enzyme bound Mn(II) was blocked. By observing the relaxation rates of $^1H$ and enriched $^{13}C$ pyruvate, a second coordination sphere association (6—7 Å) of pyruvate to CrATP was demonstrated. They also employed CrATP in combination with Mn(II) and Co(II) (substituted for Mg(II) at the protein metal binding sites) to calculate 17 distances from the two paramagnetic reference points by observing their effect on substrate $^{31}P$, $^{13}C$ and $^1H$ NMR signals. With models it was then possible to construct a tentative three dimensional map of the arrangement of metal ions and substrates in the active site. These data suggested that two magnesiums are involved in the enolization mechanism, one directly bound to the enzyme and one bound to the phosphoryl group of ATP.

Aspartokinase-homoserine dehydrogenase contains four subunits and, as the name implies, has two catalytic functions. The Mg(II) requiring enzyme is involved in the conversion of aspartic acid to the essential amino acid threonine, and its aspartokinase activity is modulated by threonine, a feedback inhibitor. In the aspartokinase step a phosphoryl group is transferred from ATP to aspartate to produce β-aspartyl phosphate. Of interest, then, is whether the kinase and dehydrogenase sites are distinct or overlapping and what role is played by the Mg(II). Cobalt(II) can be substituted for Mg(II) to yield an enzyme which retains 32% of its aspartokinase activity [36]. Ryzewski and Takahashi [36] oxidized Co(II) in the presence of the enzyme employing both oxygen and $H_2O_2$. Treatment with oxygen incorporated 0.39 cobalts per subunit whereas $H_2O_2$ oxidation yielded an enzyme with one substitution-inert cobalt per subunit. Complete loss of aspartokinase activity was observed whereas 60% homoserine dehydrogenase activity was retained. As pointed out earlier in this review, the Co(III) labeled enzyme was subjected to α-chymotryptic digestion which resulted in the isolation of homoserine dehydrogenase-active fragments devoid of cobalt [38]. These experiments support the presence of two topologically distinct catalytic sites for kinase and dehydrogenase activity. The peroxide oxidation was subsequently repeated in the presence of the substrates ATP and aspartate or the inhibitor threonine which lead to the formation of threonine-Co(III)-enzyme and aspartate-threonine-Co(III)-enzyme adducts and inhibition of kinase activity [37]. When aspartate and threonine are both present during the oxidation competitive incorporation of threonine and aspartate occurs. The sum of the two bound amino acids is always one per subunit suggesting (sterically) overlapping binding sites for threonine and aspartate in the vicinity of the metal binding sites. This observation is consistent with the competitive inhibition of aspartate utilization by threonine previously observed. In summary, these subsequent studies suggest that the metal ion cofactor may serve to bridge the substrate or the inhibitor with the enzyme and that the kinase active site consists of metal-adjacent overlapping binding sites for aspartate and threonine.

Takahashi used the Co(III)-substrate (or inhibitor)-enzyme complexes to study conformational changes caused by threonine binding at the regulatory site (there are two threonine binding sites per subunit) and to locate the sites responsible for aspartate and ATP inhibition of dehydrogenase activity [51]. The immobilized Co(III) affinity label at the kinase site has also been used with fluorescent analogues of ATP to estimate the distance between the kinase and dehydrogenase sites [52].

Other than the determination of the ratio of metal ion to substrate and inhibitor, there was no direct evidence for the formation of complexes. The activity studies suggested that all species involved were at or in the vicinity of the active site but complex formation was inferred. Establishing the nature of the complexes involved would be a formidable task but proteolytic excision of the presumed complexes, as already has been done with some degree of success by Takahashi and co-workers [38], coupled with synthesis and

characterization of models could contribute significantly to the solution of the problem.

It is of interest to reverse a modification, thereby demonstrating that the protein has not undergone an irreversible change in structure which may be independent of the modification attempted and could account for the alteration in activity. Hydrogen peroxide treatment of a protein could oxidize certain protein residues (see Sect. C(i)). Attempts to reduce the Co(III) in modified aspartokinase-homoserine dehydrogenase with $NaBH_4$ and thiol reagents resulted only in partial removal of the Co(III) and a slight restoration of aspartokinase activity [36,37]. Although this failure to reverse the modification was unsuccessful, it does not preclude achieving success in a different Co(III)-protein. Such a reversal appears to have been achieved for Co(III) complexes of phosphorylase *b* and myosin (vide infra).

It is important to note that these studies differ from the Cr(III)-ATP studies with the kinases in that the substitution-inert metal ion is incorporated in situ rather than first forming a Co(III)-substrate or inhibitor complex. The in situ approach which would be expected to resemble more closely the incorporation of the native metal ion, Mg(II), could lead to distinctly different products. For example, the reaction sequence could be metal plus protein followed by substrate incorporation, a situation not possible when the metal substrate complex is synthesized first.

Lardy and co-workers [53] found that CrATP and CrADP were competitive inhibitors of ATP hydrolysis by adenosine triphosphatase but noncompetitive inhibitors of inosine triphosphate hydrolysis. They stated that this behavior was consistent with two binding sites, a regulatory site which is specific for adenosine polyphosphates and a relatively nonspecific catalytic site. Pyruvate carboxylase is also inhibited by CrATP and CrADP as well as by other Cr(III)-nucleotide complexes [54]. The observed kinetic behavior is like that of yeast hexokinase, but much lower specificity towards various Cr(III)-nucleotides was noted. Unlike yeast hexokinase, CrATP does not form a kinetically stable complex with pyruvate carboxylase.

Using a procedure similar to that of Cleland and co-workers, Sperow and Butler [55] prepared a Cr(III) complex of dipolyphosphate, $CrPP_i$. They found that the complex was neither a substrate nor inhibitor of yeast inorganic pyrophosphatase. This is in contrast to previous observations where $CrPP_i$ was found to exhibit strong binding to several kinases [46]. It was postulated that in the kinases, metal complex interaction is through the phosphate portion of the complex while in pyrophosphatase, binding is through the metal ion. In support of this postulate is the observation that various labile divalent and trivalent metal ions as well as $CaPP_i$ are inhibitory towards pyrophosphatase [56]. It was also observed that $Cr(H_2O)_6^{3+}$ slowly inactivated inorganic pyrophosphatase consistent with its substitution-inertness.

Danchin and Buc were among the first to recognize the potential of substitution-inert metal ions as probes of Mg(II) binding sites in enzymes [57].

They used electrolysis to prepare a Co(III)-AMP complex from a Co(II) solution of AMP. They found that this complex formed a substitution-inert adduct with phosphorylase *b*. Phosphorylase *b* is a normally inactive dimeric form of tetrameric phosphorylase *a* and is active only in the presence of high concentrations of AMP, an allosteric effector. Co(III)AMP presumably labels the allosteric site and inactivates the enzyme. Addition of Fe(II), $NaBH_4$ or thiol reducing agents restores activity. That other Co(III) complexes (e.g., EDTA and aspartate) have no effect on the enzyme activity, supports specific binding of Co(III)-AMP to the allosteric site.

Myosin has also been labeled with a Co(III)-ATP complex. Werber and co-workers [58] prepared a complex of Co(III) which presumably had tridentate ATP and 1,10-*o*-phenanthroline (phen) bound. Under the conditions of synthesis employed (pH 10) it was postulated that $O_2^-$ was bound to the sixth site. Competitive inhibition of myosin ATPase activity was observed on binding of the complex. Two complex units bound per myosin, consistent with the presence of two active sites. It is of interest to note that binding of one Co(III) label (presumably to one site) actually enhances the activity by a factor of 3.4 to 3.6 relative to the presence of only one unlabeled site. However, the authors did not distinguish between sequential and simultaneous labeling of the two sites. The modification was reversed by dithiothreitol, presumably through the replacement of the Co(III) ligands. Complexes in which ethylenediamine and *N,N'*-dimethylethylenediamine were substituted for phen were much less effective in inhibiting myosin. Lack of characterization of the complex, other than determination of solution stoichiometry and a G-10 Sephadex molecular weight estimation, coupled with the fact that an 80-fold excess of the complex is needed to label myosin, makes it possible that a minor component might be the inhibitor. This Co(III)-ATP complex was also found to inactivate Coupling Factor-1 from chloroplast-($CF_1$) [59] (see note added in proof).

*Synthesis and characterization of metal ion-substrate analogues.* The remainder of this section will be devoted to a summary of the work which has been reported on the synthesis and characterization of the substitution-inert nucleotide complexes which have been employed in these studies. It should be noted at the outset that to date no Cr(III) or Co(III)-nucleotide complexes have been successfully crystallized and thoroughly characterized. This drawback has been mediated in part by the fact that some chromatographic separations of the preparative solutions have been achieved.

The first use of Cr(III)-nucleotide complexes in the study of enzyme mechanism was complicated by synthetic and characterization problems of the Cr(III) analogues [60]. Cleland and co-workers were the first to isolate and partially characterize nucleotide complexes [43,44]. As pointed out earlier, most of the studies employing Cr(III)-nucleotide complexes which have appeared from other laboratories have used Cleland's syntheses or complexes supplied directly from his laboratories. It is therefore imperative to consider

in some detail the synthesis and characterization of the Cr(III)-nucleotides. The various complexes were prepared by heating the nucleotide with $Cr(H_2O)_6^{3+}$ or chloro and aquo amines at acidic pH's. The complexes were purified by adsorption on a Dowex 50 ($H^+$) strong acid column. A pH gradient was produced by using formate as eluant, and the complexes eluted, in many cases, as focused bands when their isoionic (isoelectric) points were reached. The complexes were further purified by repeating the chromagraphic step using aniline as the eluant (formate will replace $H_2O$ in the complexes on standing). CrATP prepared in this fashion could be stored in the cold for weeks. Paper electrophoresis showed the presence of only one major band for CrATP. Gel chromatography of freshly prepared CrATP showed a broad band relative to ATP with a maximum coincident with that of ATP. The stoichiometry of the various complexes was established by analyzing for total chromium, ammonia and phosphate.

A number of observations were used to support the mode of coordination of the nucleotides. From Fig. 3 the following sites on ATP are seen as potential ligators: two oxygens on the terminal $\gamma$ phosphate, one oxygen each on the $\alpha$ and $\beta$ phosphates, the two hydroxylates on the sugar, and various nitrogens on the adenine. The visible absorption spectrum of CrATP rules out nitrogen coordination (see next paragraph) and it is highly unlikely at the pH used for synthesis (3.3) that hydroxylate would coordinate. Also the six-membered rings formed by chelation to the polyphosphate would be highly favored over the much larger rings which would result if the ribose or adenine were involved.

Typical Cr(III)($d^3$) ligand field spectra were observed with maxima at ca. 600 nm ($^4A_{2g} \rightarrow {}^4T_{2g}$) and 430 nm ($^4A_{2g} \rightarrow {}^4T_{1g}$) for the aquo nucleotides and ranging from 565—515 nm and 400—380 nm in the ammines as the number of coordinated ammonias increased from two to four. In general as the number of coordinated phosphates increased the maxima shifted to longer wavelengths. The observed shifts on phosphate coordination are consistent with the difference between the maxima of $Cr(H_2O)_6^{3+}$ (575 nm, 405 nm) and the Cr(III)-bis(dipolyphosphate) complex (626 nm, 441 nm) and indicate the lower position of phosphate relative to water in the spectrochemical series. Molar absorptivities ranged from 20 to 40.

The pH behavior of the complexes was employed to help establish the mode of coordination of the polyphosphate moiety. In considering the following, the reader should keep in mind the following $pK_a$ values for ATP. Three —OH groups ($\alpha$, $\beta$, $\gamma$) have $pK_a$ values well below 3. The terminal secondary hydroxyl has a $pK_a = 6.5$ and the adenine ring protonates (at $N_1$) with a $pK_a = 4.1$. The $pK_a$ values of the base were determined photometrically for CrATP, CrCTP, and CrGTP as 3.7, 4.4, and 2.2. These values are closer to the $pK_a$ values of AMP (3.8), CMP (4.5), and GMP (2.4) than they are to those of ATP (4.1), CTP (4.8), and GTP (3.3) or the diphosphates whose $pK_a$ values are intermediate. This would be expected if the triphosphates (ATP, CTP, GTP) formed tridentate complexes with Cr(III) leaving a single

negative charge on one of the phosphates, a situation analogous to the uncoordinated monophosphates (AMP, CMP, GMP) at those pH values. More direct evidence for this mode of coordination comes from the isoelectric pH of 3.0 determined for CrATP. At this pH the base is expected to be mostly protonated ($pK_a$ = 3.7). Charge balance requires that the uncoordinated phosphate oxygen be mostly unprotonated and have a $pK_a$ somewhat greater than two. This phosphate oxygen most likely arises from the secondary —OH group on the γ-phosphate. The marked lowering of its $pK_a$ from 6.5 to below 3 can be explained by the neutralization of three negative charges brought about by coordination to Cr(III). One possibility, not considered by the authors, is chelation to the terminal phosphate, which has been suggested for a Co(III)-AMP complex [57], and either the β or α phosphate. The $pK_a$ values of ATP are close enough for it to be difficult to rule out this possibility on the basis of the available data.

DePamphilis and Cleland also found that hydrolysis of CrATP (1 M $HClO_4$, 80°C) was three times slower than ATP [43]. This would be expected if the β- and γ-phosphates formed a substitution-inert bond with Cr(III). Recently Mildvan and co-workers [47] employed NMR to obtain further evidence for tridentate ATP coordination in CrATP by determining the number of fast exchanging protons on the coordinated waters to be six from the frequency dependence of $1/T_1$.

A series of complexes was also prepared in which ATP was coordinated to Cr(III) as a bidentate or monodentate ligand [44]. Reaction of $Cr(NH_3)_4Cl(H_2O)^{2+}$ with ATP produced two complexes in about equal yields, one which eluted from Dowex 50 as a focused band at pH 3.5, followed by a diffuse band at pH 4.5. After repeating the chromatography the two compounds were found to have isoelectric points at pH 3.3 and 4, respectively. The authors observed that this behavior was consistent with the first band consisting of bidentate β,γ-coordinated ATP and the second band consisting of monodentate γ-coordinated ATP. It will be recalled that tridentate coordinated ATP has an isoelectric pH of 3.0. As the α and β phosphates are removed from the Cr(III), the $pK_a$ value of the secondary —OH on the γ-phosphate would be expected to increase. Furthermore, the authors argue that the γ-phosphate must be coordinated in both the bidentate and monodentate ATP complexes; otherwise the isoelectric pH values would be around 5. This assumes that the secondary hydroxyl $pK_a$ would still be above 5 since the $pK_a$ of the protonated adenine nitrogen is around 3.7. Oddly the visible spectra of the two compounds are identical. This should not be the case since the authors had observed small but definite shifts in the absorption maximum and the molar absorptivity in going from tridentate ATP to bidentate ADP as would be expected on replacing phosphate with water and altering the degree of chelation. Again, the reviewer feels that the $pK_a$ values are close enough for the variations in the isoelectric points between complexes to be interpreted in more than one way. Crystallization followed by chemical characterization is essential. Preparation and characterization of diamagnetic

Co(III) analogues could be of value if NMR spectra can be adequately interpreted. Recently Cleland and co-workers have reported the preparation and characterization by $^{1}H$ and $^{31}P$ NMR of $Co(NH_3)_4ATP$ complexes [61]. If adequate crystals can be obtained, crystal structures are imperative.

The structures of the Co(III) nucleotide complexes which have been employed in protein studies are much less certain. The first Co(III)-nucleotide complex to be used was prepared electrolytically from Co(II) and AMP [57]. That a diamagnetic Co(III) complex had been produced was apparent from the high resolution $^{1}H$ NMR spectrum obtained. Chelation through two phosphate oxygens and N(7) of the adenine ring (Fig. 3) was postulated. It is clear that some kind of chelation would have to occur if such a complex were to have any chance of being stable. The absorption spectrum obtained, a very broad band at 560 nm with $\epsilon = 2800$, is very uncharacteristic of a mononuclear Co(III) complex which would involve simply AMP coordination. A limited amount of information was supplied on the characterization of the Co(III)-(phen)-(ATP) complex used in the myosin studies [58]. The solution composition and an estimate of the molecular weight determined by G-10 Sephadex chromatography were reported. The absorption spectrum is nondescript starting at 600 nm and increasing with four slight shoulders to a maximum below 300 nm.

A preliminary spectroscopic study of the complexing of nucleosides and nucleotides by Co(III) was reported by Suzuki et al. [62]. They dissolved $CoCl_3$(dien) (dien = diethylenetriamine) at various pH values, added excess amounts of various nucleosides and nucleotides, and examined the CD and visible absorption spectra of the resulting solutions after 5 to 6 h. Whereas very weak or no CD spectra ($\Delta\epsilon < 0.1$) were observed at pH 4 most of the nucleosides and nucleotides exhibited CD maxima in the visible region with $\Delta\epsilon$ between 0.5 and 1.0 at higher pH values (8 and 12). The authors postulated that this represented direct binding of Co(III) to the 2′- and 3′-hydroxy groups of the ribose which is the source of optical activity. To support their conclusion they noted that when one or both of the hydroxy groups were missing, as in 2′-deoxyadenosine, very weak CD spectra were observed at all pH values. An attempt was made to correlate the absorption spectral band maxima with the mode of coordination. However, since the degree of hydrolysis of $CoCl_3$(dien) was not determined, these latter results are of little value since the number of coordinated chlorides would drastically alter the absorption maxima due to the significant difference between $Cl^-$ and oxygen containing ligands in the spectrochemical series.

Of particular importance to studies involving Co(III), and possibly Cr(III)-nucleotide complexes, was the observation that Co(III)-dien greatly enhances (by a factor of 150) the hydrolysis of ATP at pH 4.0 and 50°C [63]. The half life for hydrolysis was reported to be 66 min.

*(iv) Binding of substitution-inert metal ions to proteins*

An important approach to enzyme modification involves the modification of amino acid side chains with functional groups which will convert these residues to chelating agents. Isomorphous replacement to establish the sign of real structure factors has been extensively applied to the determination of the crystal structure of proteins [64]. Heavy metal ions are normally employed. However, the specific binding of metal ions to proteins still remains, for the most part, an art, and the inability to prepare these derivatives has been known to delay structure determinations for several years.

One approach to the systematization of multiple isomorphous replacement has been suggested by Vallee and co-workers [65]. The study involved conversion of tyrosine residues to 3-aminotyrosines, thus producing chelating agents as integral parts of the protein. In a similar fashion, amidination with picolinimidate has been employed to convert amino groups (lysines, amino terminal residues) to picolinamidines which also function as chelating agents [66].

Clearly the most significant study to date involving residue modification to form a metal-binding functional group, was the specific modification of tyrosine-248 of carboxypeptidase A (CPD) with diazoatized *p*-arsanilic acid to give arsanilazotyrosine-248 carboxypeptidase A, [(DAA-CPD)Zn], (Fig. 4) [67]. This site-specific modification which results in an enzyme which still exhibits peptidase and esterase activity, has proven to be an unusually sensitive spectral probe of enzyme conformation and has revealed a significant structural difference between the crystalline [20] and solution states of the enzyme [68]. The study bears importantly on the proposed mechanism of action for CPD, especially the role of Tyr-248 in catalysis. Auld and Holmquist have found evidence indicating that this residue may be involved in peptidase, but not esterase, activity [69], while others argue that hydrolysis of esters and peptides should proceed by the same mechanism [70]. The role of Tyr-248 in catalysis remains obscure.

In order to test these hypotheses the modification of Tyr-248 in such a way that it could not participate in catalysis was proposed [71]. Thus, for example, if esterase activity remains and peptidase activity is eliminated, Tyr-248 is most likely required for peptidase, but not esterase, activity. The modification most likely to succeed is a complex in which the phenol oxygen

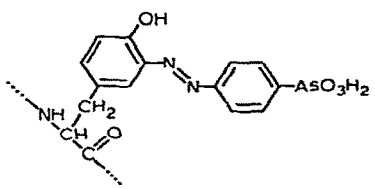



Fig. 4. Modified portion of arsanilazotyrosine-248 carboxypeptidase A.

of Tyr-248 forms a kinetically inert bond to a metal ion, such as a complex with Co(III), thus preventing its participation in catalysis. Because a cobalt-(III) complex with monodentate phenol is not likely to form readily (some type of chelation and/or relatively strong field ligands are needed to stabilize Co(III)), modifications which convert tyrosines to chelating agents are being investigated. It has been known for some time that diazonium salts will react with proteins [72], and in the case of carboxypeptidase A specific bidentate [67] and tridentate [73] arylazo derivatives of tyrosine have been prepared as discussed above.

In support of studies of cobalt azoenzyme complexes, analogue azophenol complexes have been investigated to establish criteria for the formation of cobalt complexes with such protein bound chelating groups [71,74]. These model studies are essential to the enzyme studies, because the normal visible spectral methods for determining the oxidation state of cobalt in a complex are not applicable. The azophenol ligand chromophores have molar absorptivities in excess of $10^4$ in the visible region, two orders of magnitude greater than the molar absorptivities associated with $d$—$d$ transitions. This means that spectral changes in the ligand chromophore which accompany formation of cobalt(II) and cobalt(III) azophenol complexes must be related to the oxidation state of the cobalt by magnetic methods, such as NMR and magnetic susceptibility measurements, which are much more easily performed on analogue complexes than on enzymes. Although ESR measurements below 20 K on an enzyme-cobalt complex will establish the magnetic state of the cobalt [9], easily applied visible spectral criteria greatly facilitate the studies.

These studies have shown that a systematic change occurs in the azophenol spectrum of the models on complexation to Co(III) which are distinct from the changes observed on complexation to Co(II) and Zn(II) [71,74]. These model studies are not only proving to be valuable in the characterization of the corresponding protein modifications but are playing an important role in establishing the precise conditions (pH, concentration, temperature) needed to obtain the desired protein modifications [75].

*(v) Other applications*

Included in this section is a summary of studies which cannot be classified in the above categories or for which only brief preliminary reports have appeared. These include studies of biological function in which substitution-inert metal complexes are not directly bound to the biological molecule but for which the property of substitution-inertness is required, e.g., the study of outer-sphere electron transfer processes. These studies are included in this review so that the reader will be aware of the names of those who are working in certain areas for future reference as well as the potential and breadth of application of the property of substitution-inertness.

Wherland and Gray have used substitution inert complexes such as Co-(1,10-phenanthroline)$_3^{3+}$ and Ru(NH$_3$)$_6^{2+}$ to investigate the factors influenc-

ing electron transfer in horse heart cytochrome *c* in terms of the Marcus theory of outer-sphere electron transfer [76]. By employing substitution-inert complexes they could ensure that an outer-sphere mechanism would be operative since electron transfer occurs more rapidly than ligand exchange. Their data suggest that the mechanism of electron transfer involves attack of the electron transfer agent at the exposed region of the heme and that the effectiveness of the penetration of the reagent and transfer of the electron is governed by the hydrophobicity of the reagent and its ability to attain effective $\pi$-orbital overlap with the heme. Purcell and Erman have conducted a similar study with cytochrome *c* peroxidase [77]. Dickinson and Chien have studied the iron cytochrome *c*—cobalt cytochrome *c* electron exchange reaction as a function of temperature and ionic strength in an attempt to shed some light on the electron transfer mechanism in native cytochromes [78]. These latter studies are not dependent on the substitution-inert nature of the metal ion.

Norden and Tjerneld have studied the interaction of racemic tris(bipyridyl)-iron(II) (Fe(II), $d^6$, forms spin-paired substitution-inert complexes in the presence of strong field ligands) with DNA [79]. They observed preferential binding of one of the enantiomers. The circular dichroism spectrum of this enantiomer corresponds to the isomer whose absolute configuration (defined by the spiral nature of the chelate rings) has the same chirality as DNA.

Rose and Westhead have used both cobalt and chromium as labels of the metal binding sites ($Mg^{2+}$) in enolase [42], an enzyme in the glycolytic pathway which produces a high energy phosphate bond by converting 2-phosphoglycerate to phosphoenolpyruvate. Oxidation of $Co^{2+}$ (which activates the enzyme) and $Cr^{2+}$ produces an inactive enzyme which retains the metal labels on extended dialysis. Chymotriptic digestion of the enzyme produced Co(III)-labeled peptides as previously mentioned.

Raymond and co-workers are investigating analogues of biological iron transport compounds [80]. The high-spin, labile ferric ion is transported by low molecular weight siderochromes which are manufactured by microbes. These oxygen rich molecules form very stable octahedral complexes. In order to determine the effect of stereochemistry, in particular optical isomerism, on the mode of transport of the siderochromes, Raymond and co-workers have prepared the Cr(III) substitution-inert analogues of the iron complexes and separated isomers. Not surprisingly they have observed selective transport of the optical isomers isolated.

Recently additional studies of Co(III) and other metal ion substituted transferrins have been reported [81]. However, the studies were not concerned with the property of substitution-inertness.

Finally, it is of interest to note that there is evidence which suggests that a relatively small Cr(III) complex plays a role in glucose metabolism, perhaps through some interaction with insulin. This so called glucose tolerance factor, GTF, has been isolated in very small quantities but thusfar has proven to be very unstable in its isolated form [82].

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NOTE ADDED IN PROOF

In a recent study [M.M. Werber and A. Danchin, FEBS Lett., 73 (1977) 105] it has been shown that myosin ATPase can be inactivated by in situ oxidation of Co(II)-ATP with $H_2O_2$. The inactivation pattern observed was biphasic, i.e. similar to that obtained previously using the presynthesized Co(III)-(phen)-ATP-$O_2^-$ complex [58]. These investigators ascribe the potency for inactivation to the relatively facile exchange of the superoxide ion in the latter complex. A study of the structural and exchange properties of this complex has recently been performed (J. Granot, M.M. Werber and A. Danchin, Bioinorg. Chem., in press).

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