Copper Proteins and Copper Enzymes

Volume II

Editor

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PREFACE

These volumes of *Copper Proteins and Copper Enzymes* are intended to describe the contemporary spectroscopy and other biophysical chemistry now being applied to copper proteins in order to determine the structures of their active sites. Several chapters of the treatise describe the functional understanding which is emerging from the new work. The authors are all major contributors to research progress on copper proteins and the volumes will be found to be definitive and authoritative.

The subject, copper proteins and copper enzymes, is a very lively one and is best considered in the broadest biological and chemical contexts as it continues to develop. Copper itself, born in the dust of the cosmos and comprising perhaps 0.007% of the earth's crust, occurs in the biosphere as about two dozen families of copper proteins which serve to transport O_2, to activate it toward reaction with organic molecules, and to transfer electrons between donors and acceptors. The families of copper proteins include the azurins, plastocyanins, metallothioneins, superoxide dismutases, ceruloplasmins, laccases, ascorbate oxidases, cytochrome *c* oxidases, monoamine oxidases, diamine oxidases, galactose oxidase, hexose oxidase, urate oxidases, polyphenol oxidases, phenol *o*-hydroxylases, *p*-coumarate 3-monooxygenase, dopamine β-monooxygenase, lysine protocollagen oxygenase, quercetin 2,3-dioxygenase, and the arthropodan and molluscan hemocyanins. The number of types of copper-binding domains in these proteins appears to be very limited, perhaps three or four (blue type-1 mononuclear copper sites; type-2 mononuclear copper sites; diamagnetic binuclear type-3 copper sites), and it is probable that there are very few evolutionary prototypes from which the existing families sprung.

The structures of the copper-binding domains are being rapidly worked out, as these volumes will demonstrate. However, the overall three-dimensional protein structures are in general not known, although progress is being made. The three-dimensional structures will represent major opportunities to understand the chemical biology of these proteins because their functional properties almost certainly depend upon the presence of structural domains other than the copper-binding ones; e.g., the affinity of the copper sites for oxygen may be strongly affected by the kind and disposition of amino-acid residues. In the case of hemoglobin, almost 90 abnormal hemoglobins are known to arise from amino-acid substitutions, which show altered O_2 affinities. Since the K_m (O_2) of copper enzymes is an adaptive property, it is likely that the structures of the active sites will vary in space even though the ligands remain the same for each type.

Another interesting problem against which the information in these volumes should be weighed lies in the fact that each of the functions served by copper proteins is also served by families of iron-, heme-, and flavin-containing proteins. Why then was copper selected when other prosthetic groups were available?

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He was born in Louvain in 1920 and educated there. He received his doctorate in physical chemistry (with Professor J. C. Jungers) from the Katholieke Universiteit in 1942. He was trained in protein chemistry as a Research Assistant and Senior Research Assistant of the National Fund for Scientific Research (Belgium) at the Laboratorium voor Biochemie in Louvain under Professor P. Putzeys. As a Graduate Fellow of the Belgian American Educational Foundation he was a Research Fellow in Physical Chemistry at the Department of Physical Chemistry, Harvard Medical School, Boston, Mass. (Professor E. J. Cohn, Professor J. T. Edsall, Professor J. L. Oncley).

He was President of the Vlaamse Chemische Vereniging and of the Belgische Vereniging voor Biochemie — Société Belge de Biochimie. He is a fellow of the American Association for the Advancement of Science, a member of the Royal Society of Sciences of Uppsala, Sweden, the New York Academy of Science, the American Chemical Society, the Biochemical Society (London), the Société de Chimie biologique (Paris), the Society of the Sigma Xi. He is member of the Advisory Board of the *European Journal of Biochemistry* and of *Inorganica Chemica Acta*, Bioinorganic Chemistry, Articles and Letters.

His major research interests, which included milk and barley proteins, are focused now on copper proteins, mainly on the structure, function, and biosynthesis of hemocyanins.

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COPPER PROTEINS AND COPPER ENZYMES

René Lontie

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Chapter 1

GALACTOSE OXIDASE

Daniel J. Kosman

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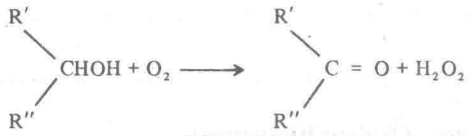
I. INTRODUCTION

Galactose oxidase (EC 1.1.3.9), a mononuclear Cu(II) protein of the nonblue or type-2 classification,[1-5] was first isolated in crude preparations by Cooper et al. in 1959.[6] It appears to be one of a class of alcohol oxidases synthesized (and usually secreted) by a variety of fungal genera.[7] These enzymes include glucose oxidase, a flavoprotein, and other essentially uncharacterized proteins.[8,9] All catalyse the reaction:



(1)

As a class, these enzymes exhibit a broad substrate specificity, e.g., primary and secondary, alkyl and aryl, other unsaturated alcohols can all be substrates for one or more of these enzymes. Galactose oxidase is the best characterized of this group largely because although a mononuclear Cu(II) protein containing no other prosthetic group, it catalyses the 2-e^- redox reaction at the substrate level using O_2 as noted above. How the enzyme catalyses this reaction is a chemically fundamental and challenging question.

II. HISTORICAL BACKGROUND

Following the identification by Cooper et al.[6] of an oxidase activity towards galactose in the liquid growth medium of a basidiomycete, typed as *Polyporus circinatus* Fr., the work on galactose oxidase was associated with Bernard Horecker's lab.[2,3,10-13] Included were a number of scientists who continued working on this enzyme after they became independent investigators, e.g., Drs. C. Asensio,[7,14,15] D. Amaral,[16] and G. Avigad.[17-21] The latter, in particular, has contributed much to the literature on galactose oxidase. The major contributions by the Horecker group were

1. The identification of the products of the reaction — H_2O_2 and galactosehexodialdose
2. The hexose specificity
3. The general method for fungal growth and enzyme purification
4. The identification of galactose oxidase as a Cu(II) protein

During this period, the question of the precise typing of the fungal species producing galactose oxidase was addressed;[22] it was argued that the organism actually synthesizing the enzyme was *Dactylium dendroides* (Northern Regional Research Lab. No. 2993, Peoria, Ill.) a fact later reconfirmed.[23] Related to this was the observation that fungal genera secreted *either* galactose or glucose oxidase, but not both.[7] In addition, optimum growth conditions for *D. dendroides* and the synthesis and secretion of galactose oxidase were studied.[17] Another important observation was also made, that is, that fungus grown in the virtual absence of medium copper secreted an apo-galactose oxidase protein in normal amounts. This apoenzyme could be activated simply by adding $CuSO_4$ to the cell-free growth medium.[17] Later workers have been indebted to this fundamental research.[24,25]

Although little research on the mechanism of action of galactose oxidase was reported before 1973, a number of studies on the assay and specificity of the enzyme were published. Although manometric,[6] Clark-type O_2 electrode,[16] and amperometric[26] techniques had all been used, most research employed a coupled assay using horseradish peroxidase (HRP)

Table 1
GROWTH MEDIUM FOR *Dactylium dendroides*

Salts, nitrogen source (A)	Metals (B)	Carbon source (C)
219 g Na_2HPO_4 (62 m*M*)[a]	10.7 g $MgSO_4 \cdot 7H_2O$ (1.6 m*M*)	Starter culture
212 g KH_2PO_4 (62 m*M*)	50 mg $MnSO_4 \cdot H_2O$ (12 μM)	5.4 g glucose (55 m*M*) in 68-mℓ H_2O
26 g NH_4NO_3 (13 m*M*)	77 mg $ZnSO_4 \cdot 7H_2O$ (11 μM)	(2 × 34 mℓ in 50-mℓ flasks)
51 g $(NH_4)_2SO_4$ (15 m*M*)	40 mg $CaCl_2 \cdot 2H_2O$ (11 μM)	
19 g NaOH (19 m*M*)	40 mg $FeSO_4$ (11 μM)	or
19 g KOH (14 m*M*)	67 mg $CuSO_4 \cdot 5H_2O$ (11 μM)[b,c]	250 g sorbose (55 m*M*)
20.4 ℓ H_2O	2.57 ℓ H_2O	2.5 ℓ H_2O
Autoclaved as 1.0 ℓ in 20 × 2-ℓ flasks and 200 mℓ in 2 × 500-mℓ flasks	Autoclaved as 250 mℓ in 10 × 500-mℓ flasks and 34 mℓ in 2 × 50-mℓ flasks	Autoclaved as 250 mℓ in 10 × 500-mℓ flasks
Thiamine stock solution; 337 mg/10 mℓ (5 μM) — not autoclaved; prepared in sterile H_2O and kept frozen between use		

[a] Concentrations in final growth medium prepared as described in Reference 35.
[b] Adding the equivalent ^{63}Cu as $^{63}Cu(NO_3)_2$ prepared by dissolving ^{63}Cu metal (powder or shot, Alfa Inorganics) in 1 *N* HNO_3 and diluting yields isotopically pure [^{63}Cu]enzyme for magnetic resonance studies.
[c] Use of AnalaR® salts (BDH, obtained from Gallard-Schlesinger) or equivalent and omission of $CuSO_4$ yields a Cu-free medium. Fungal growth is not affected while an equivalent amount of galactose oxidase is secreted as an apoprotein.[24,25]

and a peroxidase chromogen substrate.[11,13,27] This assay was adapted from that used initially for glucose oxidase.[28] A variety of chromogens were suggested (*o*-dianisidine, *o*-cresol, *o*-tolidine), all of which are carcinogens. Furthermore, they may inhibit galactose oxidase.[29] More recently, it has been demonstrated that HRP activates galactose oxidase.[30,31] Thus, the coupled assay has a variety of drawbacks which should be taken into consideration when it is used. The hexose specificity was explored in more detail;[32] dihydroxyacetone was found to be the best substrate yet reported.[16]

In addition to basic studies on the enzyme, work was progressing on the use of galactose oxidase in applied research. It was incorporated into the Galactostat® kit (Millipor Corp., Laboratory Products Division, Freehold, N.J. — similar kits are sold by a number of other biochemical supply houses) which also contained HRP and a chromogen (*o*-tolidine). This mixture is used to assay for galactose and galactose as nonreducing terminus (as in lactose) in biological fluids. Galactose oxidase also found widespread use in the oxidation of terminal galactose residues in glycoproteins exposed when the protein was desialylated with neuraminidase. The resulting C-6 aldehyde can be reduced with sodium boro[^{3}H]hydride and the radiolabeled glycoprotein used in in vivo and in vitro studies on the binding and plasma clearance of such proteins.[33,34]

III. FUNGAL GROWTH AND GALACTOSE OXIDASE BIOSYNTHESIS

D. dendroides grows readily on either agar or in liquid media supplemented only with inorganic sources of P, S, and N, normal salts and trace metals, and thiamine.[17,24,25] A typical medium is described in Table 1 which is based on earlier work.[17,24,25,35] Large-scale growth can be carried out in shaker-flasks or fermentor. In either case, vigorous agitation (aeration) is required as is the maintenance of temperature between 18 and 25°C. Also, in either case, large-scale growth is initiated using liquid inoculum from "starter" flasks. The

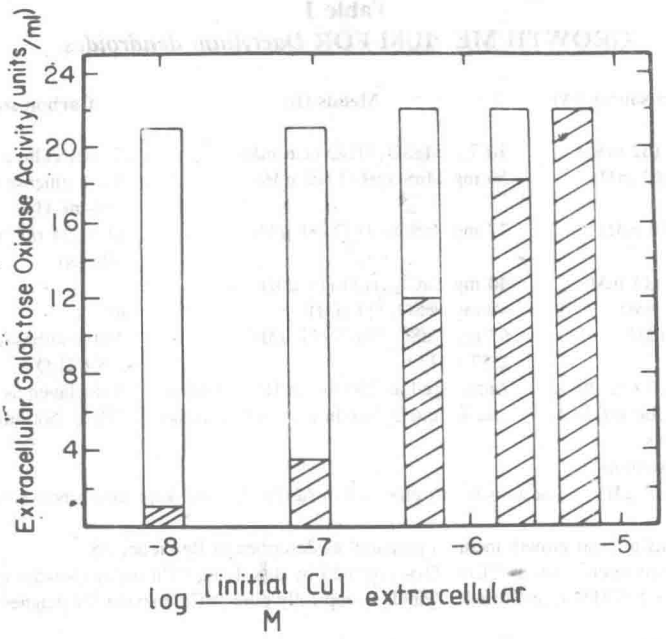


FIGURE 1. Histogram showing distribution of galactose oxidase protein between holoforms (hatched) and apoforms (open) as function of medium-copper concentrations. Apoprotein is detected by enzymic activity assay of cell-free media following addition of 0.1 m*M* $CuSO_4$.

fungal strain is maintained on 1.5% agar slants prepared in the basic medium used for these starter flasks.

The most complete study on the growth characteristics of *D. dendroides* in liquid media showed that the best yield of galactose oxidase was obtained using L-(−)-sorbose as the carbon source.[17] Although D-glucose (dextrose) was also an effective substrate, its use led to less enzyme production. However, it *must* be used in starter flask growth; inocula grown initially on sorbose exhibit altered morphology and produce no galactose oxidase. Galactose failed to provide a medium conducive to enzyme production.[17]

The pattern of mycelial growth and enzyme secretion has been examined in some detail.[17,24] Generally, a 0.5 to 2% w/v inoculum grows to a stationary phase in ≈5 days at 20°C. Significantly, cell density, which increases with the size of the inoculum[17,24] or at pH values below 6.7,[24] markedly affects galactose oxidase synthesis and secretion. Enzyme production at pH 6.0 is ≈60% of what it is at pH 7.0.[24] This behavior is one rationale for a totally artificial pH 7.0 medium (Table 1), which lacks yeast extract; fungal metabolism of the latter leads to a pH depression which shuts off enzyme production.[24] Although under some conditions some galactose oxidase is retained within the mycelia, the enzyme activity is normally found only extracellularly.

Of some interest has been the relationship between fungal copper nutriture and galactose oxidase synthesis.[17,24,25] Growth of *D. dendroides* in liquid media is independent of copper concentration (<10 n*M* to 1 m*M*). The pattern of galactose oxidase synthesis over this range of medium copper is indicated in Figure 1. As the data indicate, at [Cu] > 1 μ*M*, all enzyme protein is present in the active holoform. Below this level a steadily increasing fraction of the protein is secreted as an apoprotein. This apoprotein is readily activated by the addition of Cu(II) and is immunologically cross-reacting with antibody to the holoenzyme (Figure

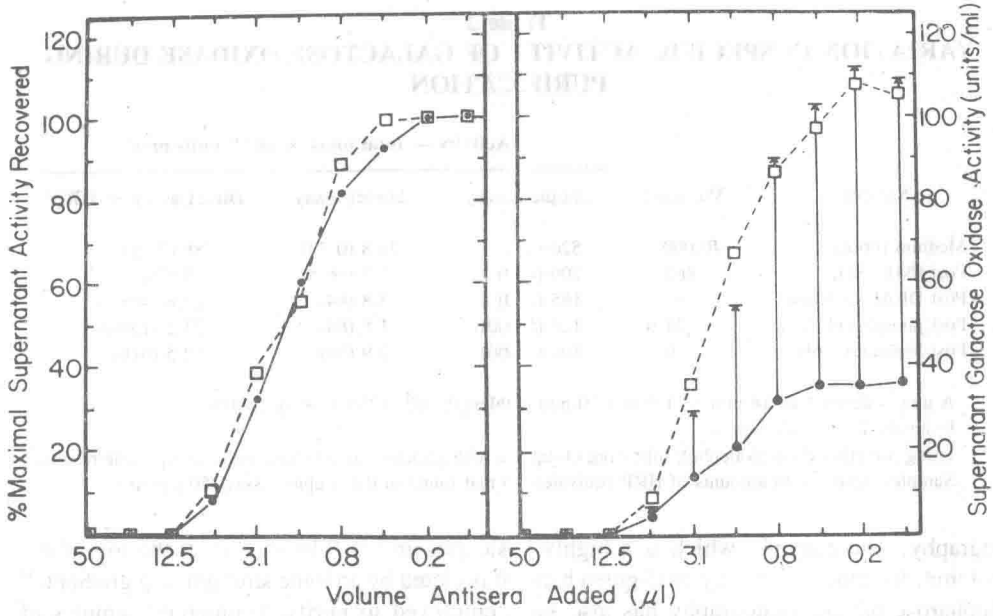


FIGURE 2. Cross-reaction between holo-galactose oxidase antisera and apoprotein in low-copper cultures. On left, both low (●) and high (□) copper media give same antigen titer as measured by percent supernatant activity, i.e., apo- and holoforms have identical antigenicity. On right, total supernatant activity is measured before and after addition of $CuSO_4$ to cell-free low-copper media (●) as in Figure 1.

2). Correlation between intracellular copper levels and holoenzyme production over time in culture indicated that below approximately 10 ng Cu per milligram cell dry weight only the apoenzyme is secreted; above this level, the holoprotein is formed.[25] This pattern was distinct from that seen for two other copper proteins, the Cu/Zn-superoxide dismutase and cytochrome *c* oxidase.[25] The latter was independent of medium copper (undoubtedly explaining the insensitivity of *D. dendroides* to $[Cu]_{medium}$) while at limiting copper, the biosynthesis of Cu/Zn-superoxide dismutase protein was repressed (no apoprotein detected). Clearly, this organism has proved an interesting system for the study of cellular copper metabolism.

IV. PURIFICATION AND ASSAY

Galactose oxidase secreted into the (artificial) growth medium of *D. dendroides* constitutes ≈11% of the total extracellular protein.[24] Isolation of the enzyme from the cell-free medium is a relatively simple procedure.[4,13,35] Initial steps involve $(NH_4)_2SO_4$ precipitation in the presence of microcrystalline cellulose (to act as a precipitate substrate and filter-aid), suction filtration, resuspension of the filter cake in $(NH_4)_2SO_4$, and elution of the absorbed protein by a phosphate buffer. These steps were worked out by Horecker and co-workers.[12,13] In this way, 20 2-ℓ shake-flasks containing 1,250 mℓ medium each (25 ℓ total) can yield ≈250 mℓ eluate containing 60 to 80 mg galactose oxidase. Following dialysis to lower the buffer concentration to 10 m*M*, the mixture is treated with DEAE cellulose to remove a substantial portion of the contaminating protein. The DEAE cellulose is removed by simple suction filtration. The mother liquor is concentrated by pressure dialysis, dialysed against a 5 m*M* phosphate buffer, and then chromatographed twice on a phosphocellulose column.[35] The whole procedure takes less than 3 days and yields are normally >60%. That galactose oxidase is a Cu(II) protein becomes readily apparent during the phosphocellulose chroma-

Table 2
VARIATION IN SPECIFIC ACTIVITY OF GALACTOSE OXIDASE DURING PURIFICATION

Sample	Vol (mℓ)	Activity — total units × 10^{-3} (units/mℓ)[a]		
		Coupled assay[b]	Direct assay[c]	Direct assay + HRP[d]
Medium filtrate	40,000	520 (13)	28.8 (0.72)	86 (2.15)
Post $(NH_4)_2SO_4$	465	200 (430)	7.7 (16.5)	35 (76)
Post DEAE cellulose	590	165 (280)	3.8 (64)	23.6 (40)
Post phosphocellulose	21.6	125 (5,000)	1.5 (68)	23.2 (1,080)
Post Sephadex® 6B	30	100 (3,600)	2.9 (96)	18.5 (616)

[a] A unit is defined as ΔA/min = 1.0 in a 10-mm pathlength cell, 1.0-mℓ assay volume.
[b] Using HRP and *o*-dianisidine.
[c] Using 3-methoxybenzyl alcohol; following O_2-uptake with galactose as substrate yields comparable results.
[d] Samples treated with amounts of HRP equivalent to that found in the coupled assay (50 μg/mℓ).

tography. The enzyme, which is a highly basic protein,[4,18] "discs" out at the top of the column, forming a distinctly blue-green band; it is eluted by an ionic strength step gradient.[35] Sepharose-6B chromatography has also been employed to purify commercial samples of galactose oxidase[36] and can effectively replace the second of the phosphocellulose chromatography steps noted above.

During purification, enzyme activity is most conveniently followed using the HRP coupled assay described above.[13] However, as noted, this assay does introduce certain artefacts, which make it unsuitable for quantitative analysis. One example of this is given in Table 2, in which is compared the "specific activity" of the protein mixture through the purification steps outlined above as determined by two assay procedures. The direct assay involves the use of one of several aromatic primary alcohols that serve as substrates for galactose oxidase,[30,31,35,37-39] 3-methoxybenzyl alcohol.[31,35] The oxidation of this alcohol can be followed conveniently by monitoring the increase in absorbance at 314 nm at which wavelength $\Delta\epsilon$ ($\epsilon_{aldehyde} - \epsilon_{alcohol}$) is 2,691 M^{-1} cm^{-1}. Thus, in an assay volume of 1.0 mℓ (b = 1 cm), a ΔA_{314} = 1.0 is equivalent to 0.37 μmol of product aldehyde formed. The coupled assay, which uses 0.1 M galactose as substrate, is ≈10 times more sensitive than the direct chromogenic one. One absorbance unit (at 460 nm using *o*-dianisidine) is equivalent to 33 nmol H_2O_2 produced, again in an assay volume of 1.0 mℓ.[4,40]

The differences apparent in Table 2 are due to: (1) the presence of an inhibitor of galactose oxidase removed completely during the second phosphocellulose (or Sepharose-6B) column and (2) the activating effects of HRP which overcome this inhibition. Thus, HRP masks the presence of this inhibitor. Clearly, comparisons as in Table 2 are useful in monitoring subtle aspects of the purification of galactose oxidase and are necessary if the enzyme is to be used in quantitative, rigorous kinetic analysis.

Another measure of enzyme purity (and activity) is provided by comparisons between the protein UV (280 nm) and Cu(II)-associated visible absorbance *(vide infra)*. One of the contaminants removed during the phosphocellulose chromatography is visualized as a yellow band; this apparently contains some proteolytic activity and can also be removed by Sepharose-6B chromatography.[36] Failure to remove this material completely causes the eluted galactose oxidase solution to have a yellowish cast to it rather than a distinct green-blue color. This yellow contaminant contributes a background absorbance between 370 and 450 nm which "fills in" the actual enzyme absorbance spectrum in this region. Active and pure galactose oxidase is characterized by a well-defined 445-nm Cu(II)-associated absorbance peak (ϵ = 1,155 M^{-1} cm^{-1})[41] such that $A_{280}/A_{445} < 27$ and $A_{445}/A_{355} > 1.6$.[42] As discussed

in more detail below, enzyme which lacks this 445-nm transition (and the other Cu(II) transitions) normally will be inactive and be colorless in solutions >5 mg/mℓ. Significantly, *all* commercial samples that have been studied in the writer's laboratory have had very weak visible absorbance and produced essentially colorless solutions. In large measure, this is due to the lyophilization of these samples. Freeze-drying generally inactivates galactose oxidase.[35] However, samples of enzyme which are stored in frozen solution at −70°C or below are stable indefinitely. Also, such samples have been shipped on dry ice with no loss of activity or alteration of molecular (spectral) properties.

V. MOLECULAR AND SPECTRAL PROPERTIES: NATURE OF Cu(II) COORDINATION

The relative molecular mass (M_r) of galactose oxidase has been a difficult parameter to determine. In all likelihood, this is due to the tendency of the protein to adsorb to surfaces, particularly glass, quartz, and carbohydrate-based chromatography substrates. Thus, a variety of M_r values were reported, ranging from 38,000 to 75,000.[2,12,18,37,43] Subsequently, comparison of data obtained from sedimentation equilibrium, osmotic pressure, SDS gel electrophoresis, and gel-permeation chromatography experiments showed that M_r = 68,000.[4] These experiments also showed that galactose oxidase formed monodisperse, non-self-associating solutions. The enzyme's affinity for surfaces is undoubtedly due to its basicity. A pI of ≈12 has been reported,[18] which is consistent with the inability to electrofocus the protein in an acrylamide gel containing pH 8-11 Ampholines.[4]

The determination of the M_r allowed the Cu(II) stoichiometry to be determined precisely; there is 1 mol Cu/mol enzyme.[4] The exact amount of copper in a particular preparation may be less than this amount but the difference can be restored by dialysis against 1 m*M* $CuSO_4$ in 0.1 *M* sodium acetate (pH 5.5).[44] The metal can be removed by treatment with diethyldithiocarbamate.[2,4,12] The metal chelate can be removed by gel-permeation chromatography[2] or pressure dialysis.[42] The apoprotein can be readily reactivated by addition of $CuSO_4$,[2,4,12] but not by other transition metals; copper is the only metal which binds to the enzyme.

The apo- and holoproteins are conformationally similar. The $s_{20,w}$ value for the holoprotein is 4.76 S, for the apoprotein 4.83 S.[4] The far UV CD spectra for each are essentially indistinguishable; both contain only small amounts of α-helix and β-sheet with a preponderance of unordered structure.[41] Upon copper removal there are some distinct changes in the near UV CD and absorbance spectra associated with environment changes experienced by (a few) aromatic (tryptophan) residues.[45,46] This change is also reflected in alterations in tryptophan fluorescence.[47] The electrophoretic behavior is not changed by copper removal. The holoenzyme has $\epsilon_{280\ nm}$ = 104,900 M^{-1} cm^{-1} and $A^{1\%}_{1\ cm}$ at 280 nm = 15.4.[41]

However, the holoenzyme is more resistant to denaturation than is the apoprotein. The enzyme is active in 6 *M* urea.[4] Only apoprotein is susceptible to proteolytic digestion or -SH group titration (with PMB).[4] Also, only apoprotein disulfides can be reduced, presumably because these groups in the holoprotein are not exposed when the protein is treated with denaturants. Galactose oxidase contains five cysteine residues, four of which are involved in disulfide linkages in the native protein. The one free sulfhydryl group may be in the enzyme active site,[4] but is not likely to be a component of the Cu(II) chelate itself.[5,48-51]

The enzyme is isozymic (Figure 3).[4,35] There are no apparent activity differences among these isozymes. Since galactose oxidase contains no carbohydrate,[4] these charge differences cannot be due to variations in monosaccharide content. Rather, these charge variations are more likely to be associated with deamidination of asparagine and glutamine residues. The large ammonia peak noted in the amino-acid chromatogram, large content of glutamic and aspartic acid in the protein hydrolysate (Table 3), and *basic* nature of the protein itself

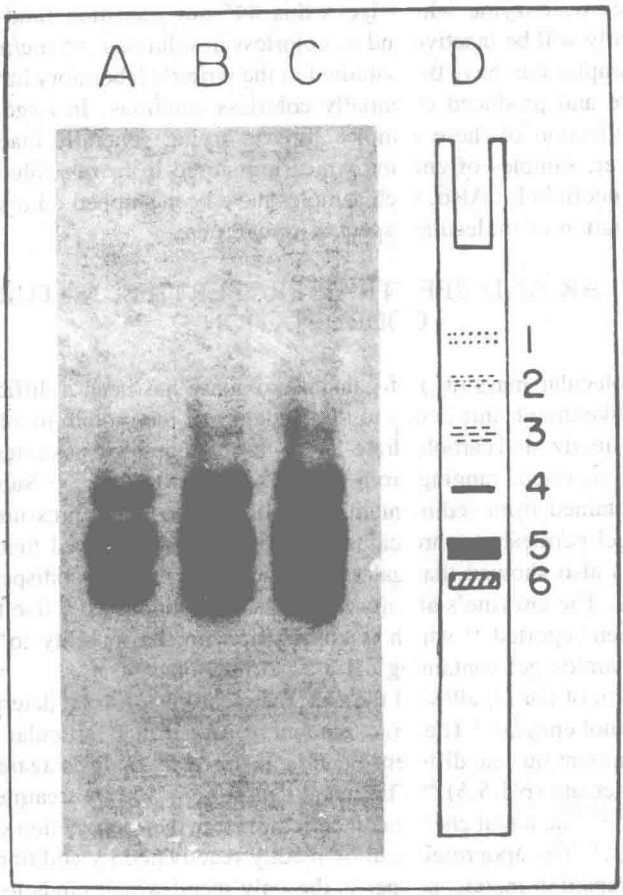


FIGURE 3. Activity stain (A-C) of galactose oxidase[52] following pulsed-power electrophoresis in 7.5% acrylamide, pH 8.6.[4] In D is indicated the pattern of protein staining using Coomassie® Brilliant Blue G. Cathode is at bottom.

indicate that galactose oxidase contains a large proportion of glutamine and asparagine. Spontaneous hydrolysis of some of the these residues would thus be responsible for the development of the isozymic pattern observed.

Galactose oxidase exhibits electronic transitions characteristic of Cu(II) proteins (Table 4).[41] The absorption coefficients and energies of these transitions put galactose oxidase into the type-2 or low-blue or nonblue class of copper proteins.[1] These transitions, together with the spin Hamiltonian parameters (Table 5) indicate that the Cu(II) center has a pseudo square-planar geometry; the lack of $g_\perp$ anisotropy has been confirmed by EPR spectra obtained at 35 GHz.[50,53] In addition, this geometry and the lack of asymmetric ligand components (i.e., thiolate coordination) are indicated by the fact that the g tensors are rather insensitive to an externally applied electric field (the [linear] electric field effect).[51] By these criteria, the Cu(II) site in galactose oxidase is similar to simple Cu(II) complexes involving O and N coordination.[1,48,51,53-55]

The identity of three of these coordinating groups has been suggested by a number of EPR experiments.[5,48,50,51,53,56] Both the $g_\parallel$ and $g_\perp$ regions exhibit superhyperfine splittings attributable to N coordination; the $M_I = 3/2$ (low field) $g_\parallel$ transition contains what can be interpreted as a five-line superhyperfine line pattern associated with two equivalent nitrogen