Title

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Author

Swanson, M.

Publication Date

1979-10-01

Lawrence Berkeley Laboratory
UNIVERSITY OF CALIFORNIA

ENERGY & ENVIRONMENT
DIVISION

Submitted to Archives of Biochemistry and Biophysics

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Maurice Swanson, Rolf Mehlhorn, Lester Packer,
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October 1979

Prepared for the U.S. Department of Energy under Contract W-7405-ENG-48

DISCLAIMER

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Received:

Maurice Swanson, Rolf Mehlhorn, Lester Packer

Membrane Bioenergetics Group

Lawrence Berkeley Laboratory

and The Department of Physiology-Anatomy

University of California

Berkeley, California 94720

and

Paul Smith

The Pierce Chemical Company Research Department

Rockford, Illinois 61105

Running Title- CYTOCHROME c-CYTOCHROME OXIDASE INTERACTIONS

Abstract

A spin-labeled imidoester has been synthesized and reacted with cytochrome c and cytochrome oxidase. Advantages of this reagent as a spin label include its specificity for primary amino groups, its water solubility, and preservation of charge at the reaction site. The spin-labeled cytochrome c exhibits obvious spectral changes upon interacting with lipid vesicles and cytochrome oxidase preparations, and is useful as a probe of binding characteristics. Spin-labeled cytochrome c in buffer has a correlation time, τ_c, of $9.10 \pm 0.18 \times 10^{-9}$ sec. When spin-labeled cytochrome c binds to c-depleted beef heart mitochondrial inner membranes or to purified cytochrome oxidase, the correlation time increases. The correlation time of cytochrome c bound to inner mitochondrial membranes is $1.92 \pm 0.30 \times 10^{-9}$ sec at an ionic strength of 25 mM (cacodylate buffer) and diminishes as the ionic strength is increased until the membrane free signal is recovered at 0.2 M KCl. The mobility of cytochrome c interacting with cytochrome oxidase preparations decreases markedly with the lipid content of the preparation. Kinetic parameters of cytochrome c-cytochrome oxidase preparations were unaltered when 1-2 amino groups of cytochrome c were modified with the spin-labeled imidoester.

Introduction

Cytochrome c interacts with the inner mitochondrial membrane by electrostatic attraction of positive charges on cytochrome c to negative charges on the membrane(1). Lysine residues comprise the majority of these positive charges and appear to play a critical role in the interaction with cytochrome c reductase and oxidase. Thus Margoliash and coworkers(2-4) have shown that modification of lysines 13, 72, 87, and 27 with charge reversing reagents led to substancial reduction of high affinity site electron transfer activity while modification of lysines 22/99, 39, and 60 led to less inactivation. Further characterization of the manner in which specific lysine residues are implicated in binding of the protein to membranes and to the active site on cytochrome c oxidase and cytochrome c reductase is of interest.

Previous experiments with spin-labeled cytochrome c have shown that electron spin resonance techniques could be used to directly observe binding of cytochrome c to inner mitochondrial membranes(5,6). We now report the synthesis and use of a lysine-specific imidoester spin label, whose advantages relative to other spin labels include its greater water solubility, specificity, and retention of positive charge at its reaction site.

Materials and Methods

Beef heart mitochondrial cytochrome c oxidase, isolated according to (7), has a heme a content of 9.0-10.5 nmoles/mg protein, a lipid content of 0.35-0.42 mg/mg protein, and activity(turnover number), performed according to (8), in the range of 300-450 electrons/sec per aa_3 unit.

Cytochrome oxidase, isolated as in (9), had a heme a content of 10.0-11.8 nmoles/mg protein, a lipid content of 0.02-0.06 mg/mg protein, and an activity of 360-420 electrons/sec. Cytochrome c concentration was calculated using a $\Delta\varepsilon_{540-550}$=20 $mM^{-1}cm^{-1}$(2), and cytochrome aa_3 using a $\Delta\varepsilon_{604-630}$=24 $mM^{-1}cm^{-1}$(9). Protein concentration was determined as in (14), and phospholipid content was calculated from a total phosphorous determination of an alkaline 2:1 chloroform:methanol extract(15). Cytochrome c(Type VI), ascorbic acid, N,N,N',N'-tetramethylphenylenediamine (TMPD), cholic acid, deoxycholic acid, and sodium dodecyl sulfate(SDS) were obtained from Sigma Chemical Co., and Tris(hydroxymethyl)-aminomethane and ammonium sulfate were from Schwarz-Mann.

Gas chromatography was performed using a glass column(6' x 1/8") packed with 10% OV-17(Ohio Valley) on Chromsorb W using a Hewlitt-Packard 80/100(initial column temperature was 200°C while the injection temperature was 250°C).

Synthesis of the Imidoester Spin Label

The preparation of the imidoester spin label is diagramed below.

Methyl-3-imidate-2,2,5,5-tetramethylpyrroline-1-oxyl hydrochloride

The method of base catalyzed imidoester formation as in(11) was used. Thus, 3.0 gm of 3-cyano-2,2,5,5-tetramethylpyrroline-1-oxyl(10) and 26 ml of 0.687 M $NaOCH_3$ in methanol were mixed, and the clear solution was allowed to stand at room temperature for 15 min. At the end of this period sodium methylate was destroyed by gassing the solution with dry CO_2 to saturation. Following filtration of the sodium methylate-CO_2 adduct, the clear solution was evaporated to dryness under a vacuum yielding a light orange oil. Gas chromatographic analysis of the reaction product revealed several impurities including a large amount of unconverted nitrile starting compound. A small sample of the reaction product(as an ether solution), when washed with 0.1 N HCl/H_2O and resubjected to gas chromatographic analysis, showed complete and selective removal of one of the reaction products which was assumed to be the desired free imidoester. On this basis, all of the remaining reaction product was dissolved in 25 ml anhydrous ether to which ether saturated with anhydrous HCl was added drop-wise(causing an orange precipitate to form) until gas chromatographic analysis of the supernatant revealed only a trace of the desired compound to remain in solution. The precipitate was filtered under nitrogen, washed with fresh ether and dried under vacuum. Yield was 700 mg. Infrared(KBr disk) revealed strong absorbance at 2860 cm^{-1} and 1625 cm^{-1} characteristic of the imdoester hydrochloride. The spectrum showed a complete absence of nitrile, however starting nitrile can be recovered from the unprecipitated supernatant. Values for the product elemental analysis(C=52.78, H=8.15, N=12.38, Cl=15.57) were similar to expected values(C=51.39, H=7.76, N=11.98, Cl=15.17)(analysis performed by Galbraith Laboratories, Knoxville, Tenn.)

It was pointed out to the authors(Professor W. Hubbell, private communication) that the methoxy adduct to the ring β carbon might also be formed under the stated reaction conditions. Subsequent reaction of this product with methanol would lead to an imidoester differing in structure from the molecule proposed here. The elemental analysis supports the interpretation that the major product is the one described in the present reaction scheme.

EPR

EPR spectra were recorded on a Varian E-109E spectrometer using a 1 mm inside diameter glass capillary cell and a quartz dewar. The temperature was controlled at 20°C with a Varian E4540 Variable Temperature Controller.

Spectral data of spin-labeled cytochrome c were analyzed in terms of correlation times, τ_c, defined as in (16):

$$\tau_c = 6.5 \times 10^{-10} w_o \left(\sqrt{\frac{h_o}{h_1}} - 1 \right)$$

where w_o is the width of the midline in gauss and h_o and h_{-1} are the heights of the mid and high field lines, respectively. Four separately prepared samples were analyzed to obtain standard deviations presented with correlation times in this report. This formula is valid in the fast tumbling range($\tau_c > 10^{-9}$ sec) and applies to a homogeneous population of spins, where the concept of a single correlation time is applicable. Even though these criteria were not met in the present study, the formula was applied nevertheless as a standard quantitative measure for comparing spectra. When differences in correlation times are stated it should be remembered that these could reflect effects at one labeling site and that other sites could conceivably remain unaltered.

Imidoester Treatment

Stock solutions of the imidoester spin label(20 mM) were prepared as in (12) except that 0.133 M dibasic potassium phosphate was used as the stock buffer. Reduced and oxidized cytochrome c(0.5 mM) was incubated for up to 24 hr at 20°C with 10 mM spin label. The degree of amidination resulting from imidoester treatment was determined by following the loss of free amino groups by using fluorescamine(12) after Sephadex G-25 (Pharmacia) chromatography in 50 mM KH_2PO_4 pH 7.5. The spin content of each sample was calculated from the second integral of the spectra using a PDP-11/34 computer interfaced with the EPR spectrometer.

Binding of Ferricytochrome c to Cytochrome Oxidase

Monomeric ferricytochrome c was bound to cytochrome oxidase in an approximately 1:1 molar ratio as in (2) except that 300 µl samples were applied to a 1.5 x 50 cm column of Sephadex G-75 at 22°C, or a 1.5 x 30 cm column at 4°C. Cytochrome c was added in a five-fold molar excess to cytochrome aa_3, which had been dialyzed against 25 mM Tris-cacodylate, 0.25% Tween 20, 0.05% cholate pH 7.8, and the resulting mixture was applied to the G-75 column.

Enzyme Assays

Cytochrome oxidase activity was assayed by polarographic determination of O_2 in 4.0 ml of buffer at 25°C according to (8) in the presence of 12.5 mM potassium ascorbate, 0.5 mM TMPD, 2.5-25 µM cytochrome c and 1% asolectin, 67 mM KH_2PO_4, 0.5% Tween 80 pH 7.4. The method of (3) was used for the determination of the dissociation constant for high affinity binding in a mixture of 7 mM postassium ascorbate, 0.7 mM TMPD, and 0.004-4 µM cytochrome c with 50 nM cytochrome oxidase in 25 mM Tris-acetate pH 7.8. In the latter case, monomeric ferrocytochrome c was obtained

by gel filtration through G-75 Superfine in a column(0.7 x 30 cm) equilibrated in 50 mM Tris-acetate pH 7.5(13).

Results and Discussion

Reaction of the spin label with cytochrome c occurs slowly with a substantial amount still reacting at 24 hr(Fig 1). Double integration of the first derivative EPR spectra showed that after 24 hr of treatment only 2.2 spins were bound per molecule of cytochrome c, while analysis of the free amino groups indicated 3.8 lysines had reacted. Measurements of the EPR spectra of the imidoester in buffer revealed a 30% overall reduction in the line height of the nitroxide over a 24 hr period at room temperature;the kinetics of this reduction were not studied. The susceptibility of nitroxides to acid-mediated reduction is well known, perhaps acid released during hydrolysis of the imidoester was responsible for the observed spin loss. Assuming a similar spin loss in the cytochrome c reaction mixture, the observed spin content agrees quite well with the fluorescamine assay.

By comparison, labeling of cytochrome c with methyl acetimidate($t_{1/2}$ hydrolysis=27 min) and methyl benzimidate($t_{1/2}$ hydrolysis=55 hr) at pH 8.5 shows that 17 and 15, respectively, of the total lysines are modified in 30 min by 40 mM reagent. Perhaps the double bond within the ring structure of the label stabilizes the imidoester and slows its reaction. Under these labeling conditions the oxidized and reduced spectra of spin-labeled cytochrome c are unchanged. SDS gel electrophoresis of the spin-labeled cytochrome c did not reveal the presence of oligomers; crosslinking by other monofunctional reagents has been hypothesized to

occur(12).

Since the imidoester labeling of proteins is specific and preserves charge, it was of interest to see if labeled cytochrome c molecules retained similar electron transfer and binding activities as the native protein. Eadie-Hofstee-Scatchard analysis(3) demonstrated that the spin-labeled cytochrome c molecules(2 spins/c) were identical to the native protein, and similar to cytochrome c extensively modified with methyl acetimidate(17 lysines modified)(Fig. 2). Chromatography at low ionic strength in the presence of cytochrome oxidase resulted in a 0.8-1.1:1 (c:aa_3, molar ratio) complex for both native and modified cytochrome c.

The spectra in Fig. 3a demonstrate that the imidoester spin label is weakly immobilized on both cytochrome c(τ_c=9.10±0.18 x 10^{-10}) and cytochrome oxidase molecules. These results are similar to those reported by Drott et al(5) using N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl-bromoacetamide to label the free sulfhydryl of yeast cytochrome c(τ_c= 1.0 x 10^{-9}) and to the results of Azzi et al(6) using N(2,2,5,5-tetramethyl 3-pyrrolidyl-1-oxyl) iodoacetamide to label methionine-65 on horse heart cytochrome c(τ_c=9.3 x 10^{-10}). However, one of the principal advantages of using an imidoester spin label for protein spin labeling is its water solubility. The weak immobilization and hyperfine coupling(16.0 gauss) indicate the nitroxide is exposed to a polar environment.

Reaction of the imidoester occurs with unprotonated lysines and is thus favored at high pH. The high positive charge density of cytochrome c tends to repel the positively charged imidoester and also raise the local pH as a function of proton repulsion. As these two effects tend to cancel each other it is difficult to predict which lysine residues on the protein

will react using the criteria of local charge density. The binding of cytochrome c to cytochrome oxidase in 25 mM cacodylate and in 50 mM phosphate was studied. In order to obtain a cytochrome c-cytochrome oxidase complex where c was only bound to its high affinity binding site, chromatography at low ionic strength was performed(2). Since this has been kinetically described as cytochrome c bound only to its high affinity site further immobilization of the nitroxide might be expected to occur if there had been substantial labeling of the lysines near the heme crevice. The spectra of the imidoester-labeled cytochrome c, shown in Fig. 3, indicated that most of the spins become more immobilized upon binding of the protein to its binding site(τ_c=2.71±0.22 x 10^{-9}). This suggests that most of the labeling has occurred at or near the binding site, or (less likely) that upon binding there is an alteration of the cytochrome c structure leading to changes in the environment of spins that are distant from the binding site. The splitting of the three nitroxide lines(hyperfine coupling) increases by about 0.2 gauss to 16.2 gauss upon binding and therefore the environment of the spins remains polar as the spins become immobilized. Hyperfine coupling measurements are approximate because of asymmetric line broadening effects in these immobilized spectra.

Binding of spin-labeled cytochrome c to a lipid-poor(9) cytochrome c oxidase preparation results in significantly less immobilization of the nitroxide that the lipid-rich preparation(7). This indicates that the cytochrome c binding sites on these two oxidase preparations are dissimilar even though they are kinetically indistinguishable at low ionic strength. Binding of spin-labeled cytochrome c to negatively-charged phospholipid vesicles also immobilizes the nitroxide but to a lesser extent than cytochrome oxidase.

The binding of spin-labeled cytochrome c(2 spins/c) to cytochrome c-depleted mitochondrial inner membranes was salt dependent(Fig. 4). The correlation time of the bound c(τ_c=2.27±0.13 x 10^{-9}) was similar to that of the bound cytochrome c specifically labeled at methionine-65(14)(τ_c= 2.3 x 10^{-9}). This indicates that most of the spins became immobilized upon binding to the salt-depleted membranes.

Acknowledgements: This research was supported by the Energy and Environment Division of the Lawrence Berkeley Laboratory U.S. Department of Energy under Contract No. W-7405-ENG-48, and by the NIH(GM-24273). M.S. was the recipient of a Chancellor's Patent Fund Grant from the University of California. The imidoester spin label is now available commercially from Pierce Chemical Co.. We wish to acknowledge Drs. W. Hubbell, G. Humphries, and J. Trudell for their useful discussions.

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Figures

Fig. 1. Time course of imidoester spin labeling of cytochrome c.

Fig. 2. Eadie-Hofstee-Scatchard plot of native, imidoester spin-labeled, and methyl acetimidate-treated cytochrome c.

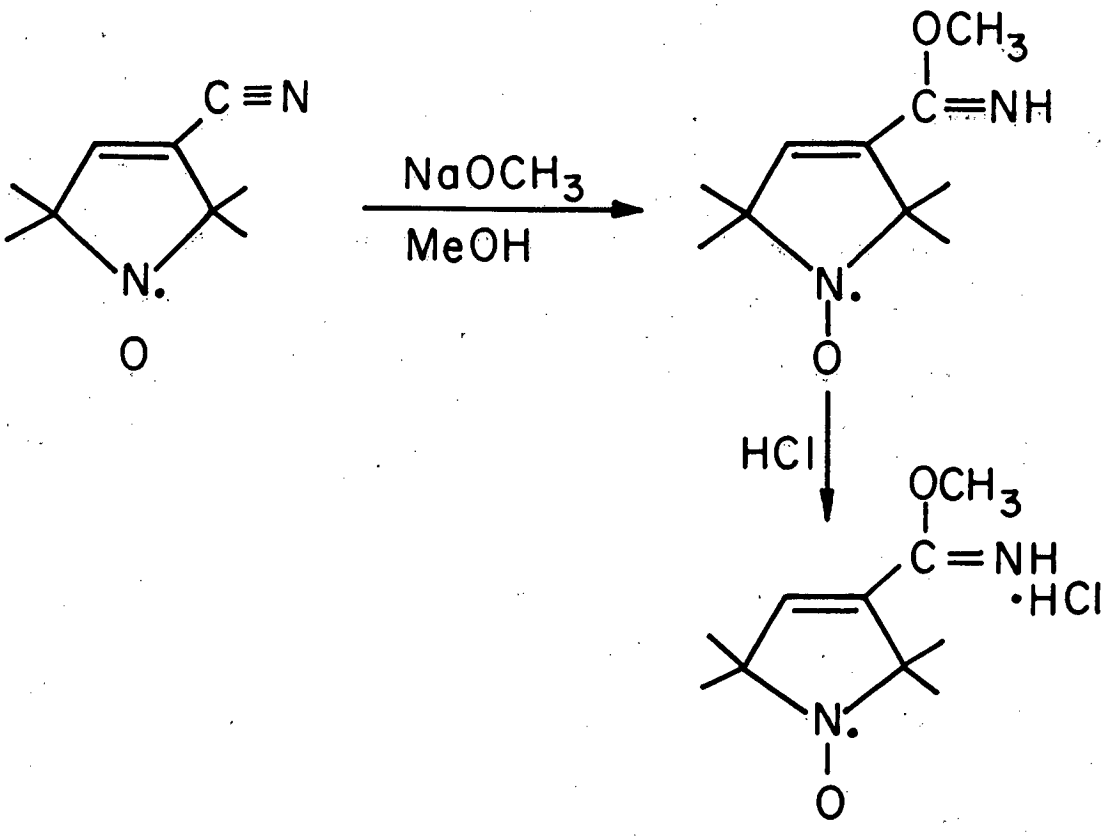
Fig. 3. ESR spectra

a) free imidoester spin label, and covalently attached to cytochrome c and cytochrome oxidase.

b) labeled cytochrome c bound to lipid-rich and lipid-poor cytochrome oxidase and asolectin vesicles.

Fig. 4. Dissociation of imidoester spin-labeled cytochrome c from cytochrome c-depleted inner mitochondrial membranes.

PREPARATION OF IMIDOESTER SPIN LABEL



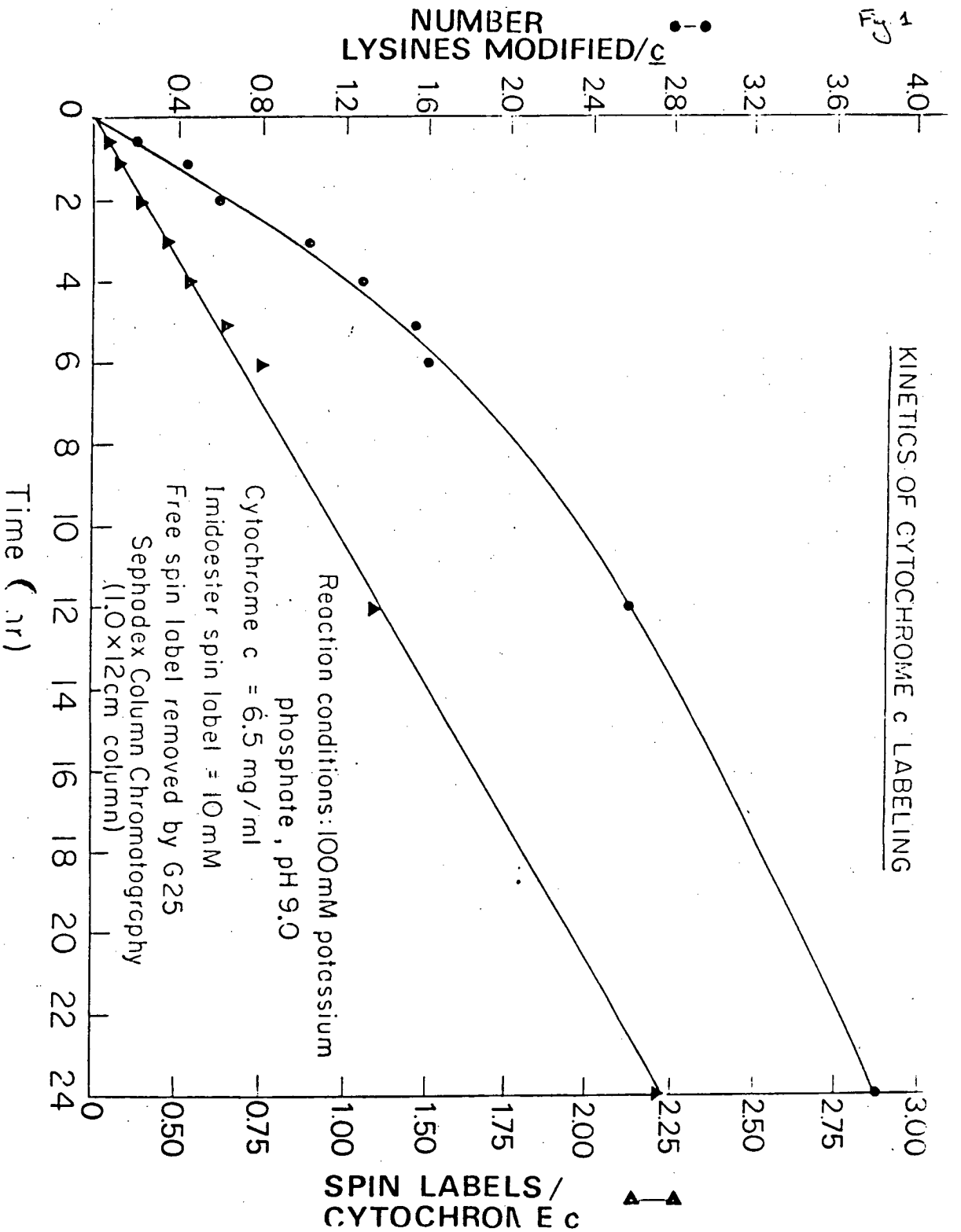
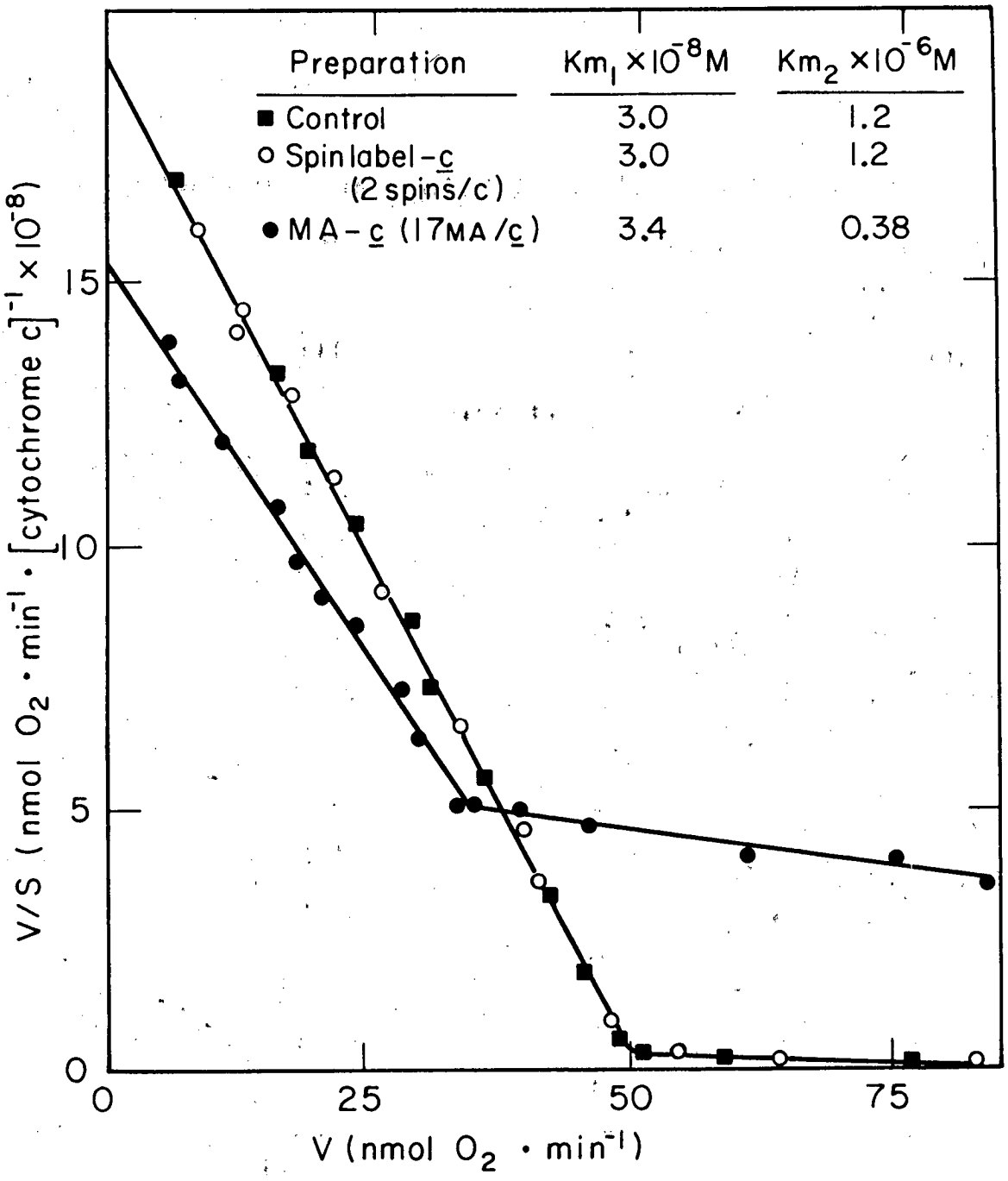

KINETICS OF CYTOCHROME c LABELING
NUMBER LYSINES MODIFIED/c
SPIN LABELS / CYTOCHROME c
Time (hr)
Reaction conditions: 100mM potassium phosphate, pH 9.0
Cytochrome c = 6.5 mg/ml
Imidoester spin label = 10 mM
Free spin label removed by G25 Sephadex Column Chromatography (1.0×12cm column)

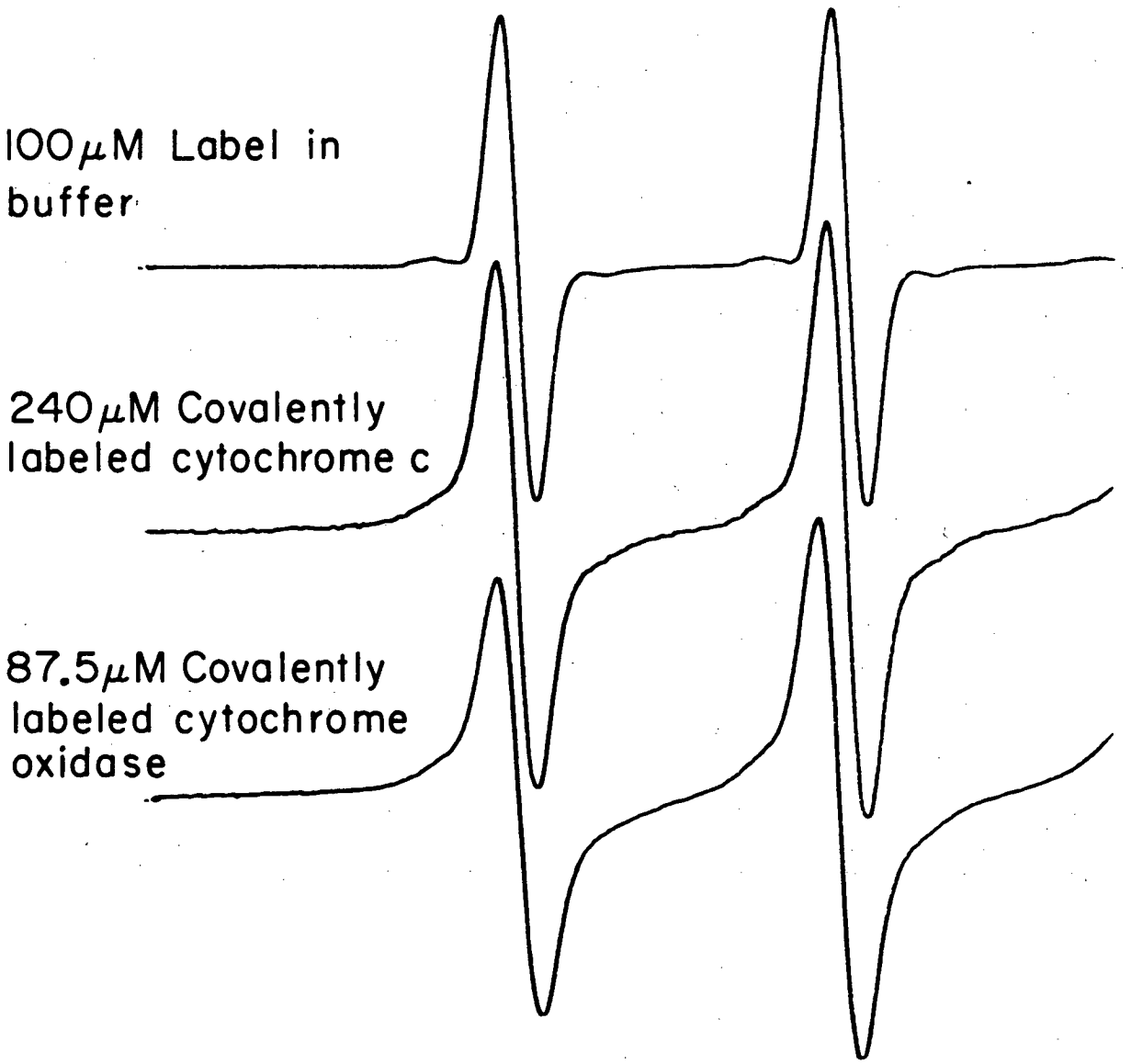
Fig 2



XBL799-3802

FIG 3A

BOUND AND FREE IMIDOESTER SI



F. 3A

:E IMIDOESTER SPIN LABEL

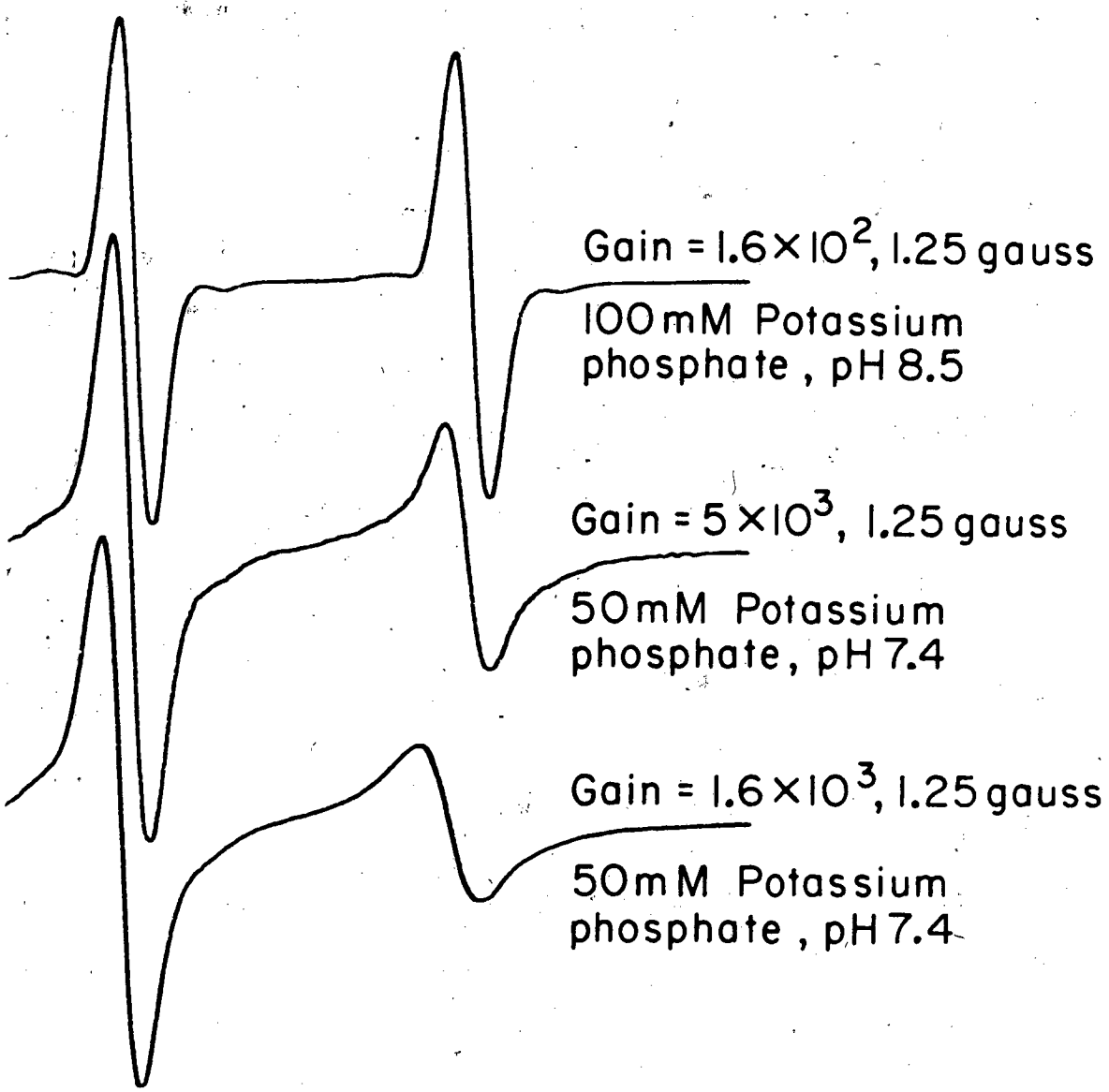


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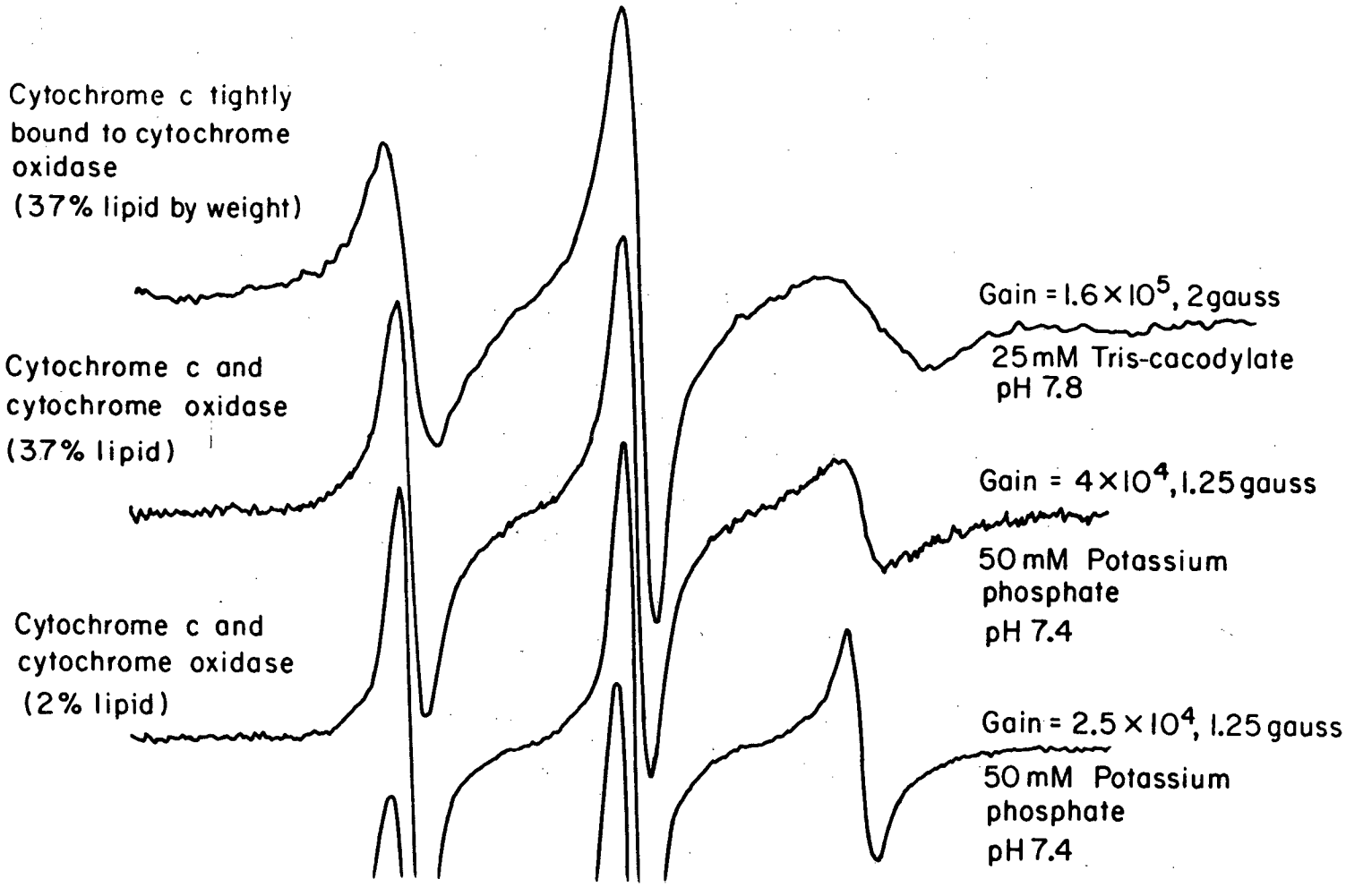
Fig 5b

BINDING OF SPIN LABELED CYTOCHROME c



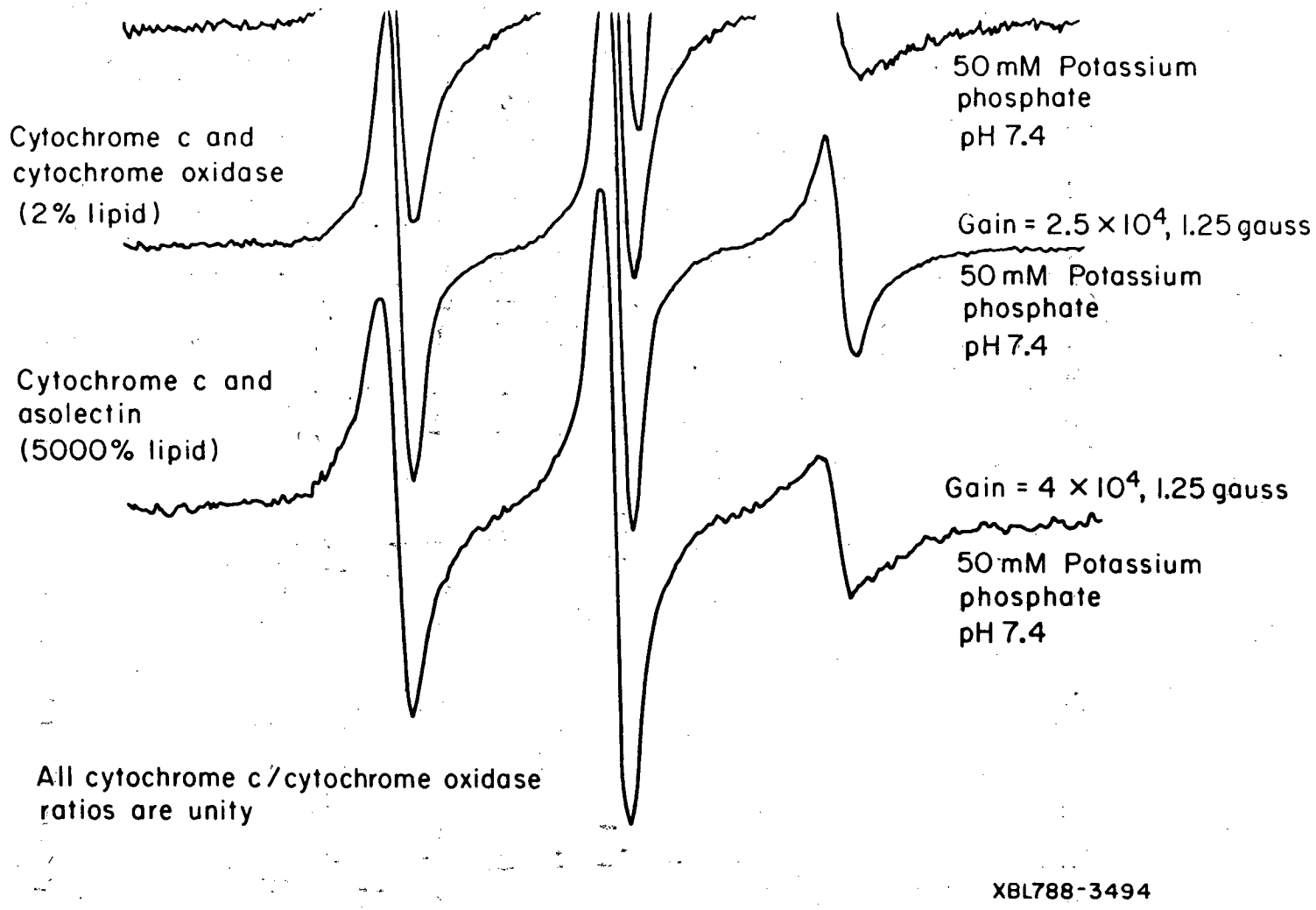
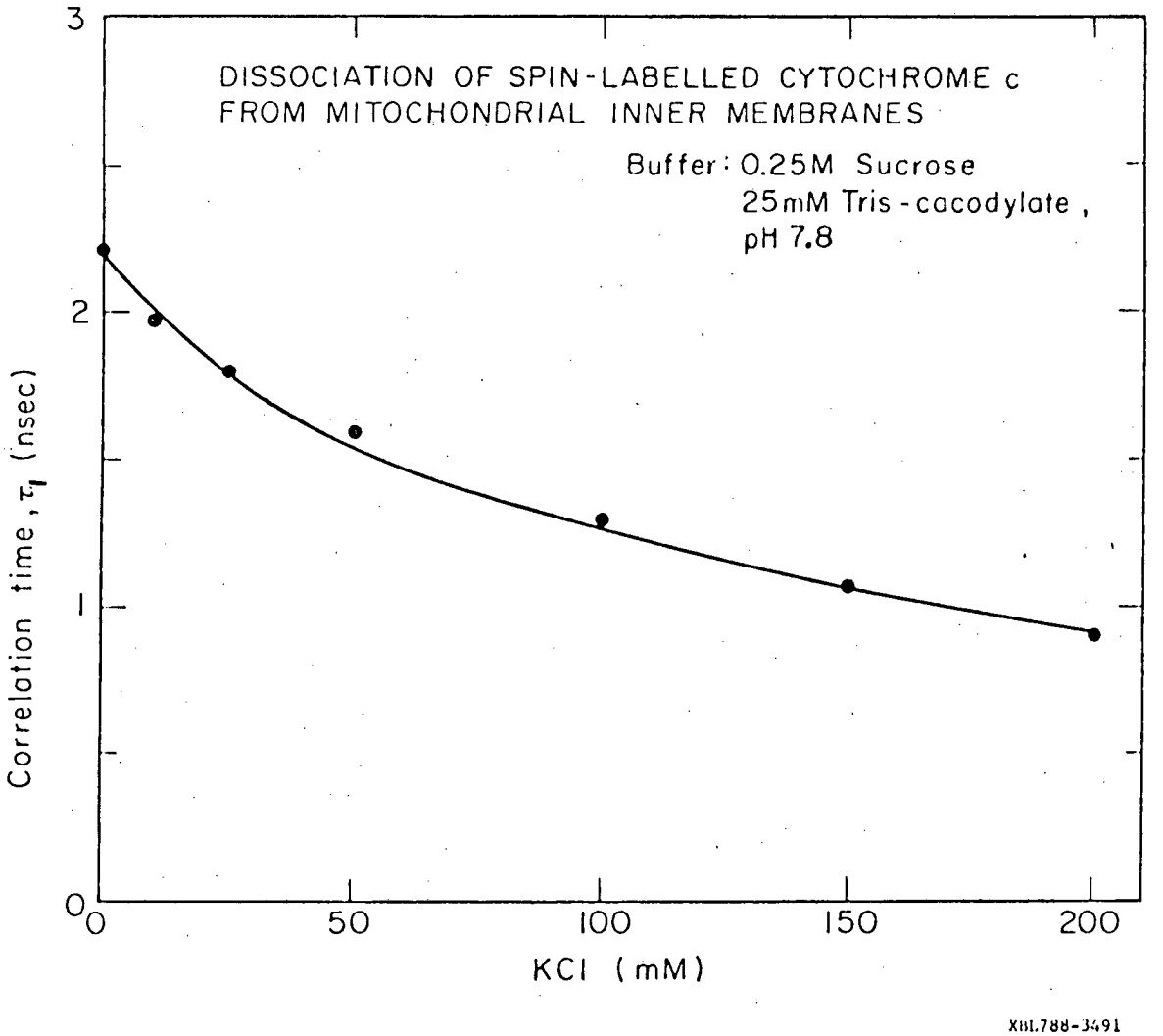

Cytochrome c and
cytochrome oxidase
(2% lipid)
50 mM Potassium
phosphate
pH 7.4
Gain = 2.5 × 10⁴, 1.25 gauss
50 mM Potassium
phosphate
pH 7.4
Cytochrome c and
asolectin
(5000% lipid)
Gain = 4 × 10⁴, 1.25 gauss
50 mM Potassium
phosphate
pH 7.4
All cytochrome c/cytochrome oxidase
ratios are unity
XBL788-3494

Fig. 4



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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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