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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

DOROTHY ANN SWAIN

ENTITLED THE PURIFICATION AND MODIFICATION WITH PHENYLGLYOXAL OF

SACCHAROMYCES CEREVISIAE CYTOCHROME C

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF BACHELOR OF SCIENCE

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THE PURIFICATION
AND
MODIFICATION WITH PHENYLGLYOXAL
OF
SACCHAROMYCES CEREVISIAE CYTOCHROME C

BY

DOROTHY ANN SWAIN

THESIS

for the

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I would like to thank everyone in the Scott group for all of your friendship, advice, assistance and tolerance.

My very special thanks go to Dr. Robert A. Scott for giving me the opportunity to work for you and for always finding the time to listen to me.

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INTRODUCTION

Cytochrome *c* is one of several cytochromes present in every eukaryote's mitochondrial electron transport chain, or respiratory chain. Respiration occurs in a controlled manner as electrons pass step-wise from one cytochrome to the next, and this process is coupled with the efficient synthesis of ATP for use by the cell. The development of cytochromes that could take part in respiration was evolutionarily significant because it allowed organisms to extract more energy from the breakdown of food molecules(1). The fundamental nature of electron transport is apparent from the fact that, so far, cytochrome *c* extracted from any organism has *in vitro* activity with the oxidase complex extracted from any other organism(2). Respiratory chain activity must have a basic mechanism that is essential to eukaryotic life.

The components of the electron transport chain are located at the inner mitochondrial membrane(3, Figure 1). Of the five cytochromes (*b*, c_1, *c*, *a* and a_3) and the two other components of the chain (ubiquinone and the NADH dehydrogenase complex), cytochrome *c* is the most accessible for study. In contrast to the others it is small and water-soluble, and it is not embedded in the membrane. It has been extensively studied by scientists interested in the mechanism of respiratory electron transport.

Primary structural data has been collected for cytochrome *c* molecules extracted from many organisms(1). In 1972 more than forty sequences had been determined and compared. Sequence homology yields two-fold information. First, the percent variation between sequences

Figure 1. The spatial arrangement of the components of the electron transport chain in the inner mitochondrial membrane. The cytochrome oxidase complex consists of cytochromes a and a_3. The cytochrome b-c_1 complex is sometimes referred to as cytochrome reductase. This figure was adapted from Alberts, Molecular Biology of the Cell, p. 507.

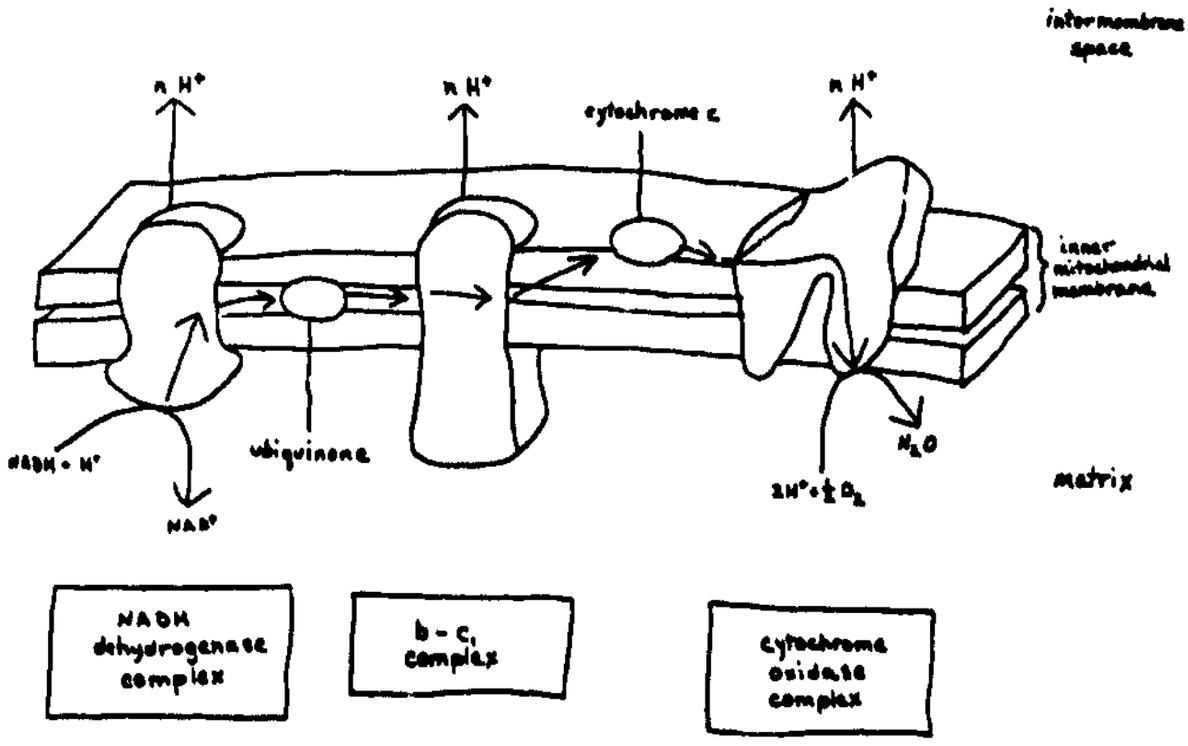
intermembrane space
n H+
n H+
cytochrome c
n H+
inner mitochondrial membrane
NADH + H+
NAD+
ubiquinone
H2O
2H+ + ½ O2
matrix
NADH dehydrogenase complex
b - C1 complex
cytochrome oxidase complex

Figure 1

from two organisms' cytochrome c can be directly related to the number of years since those organisms diverged; thus variation patterns can help to establish or confirm phylogenetic trees. Second, the pattern of conserved and variable amino acids for a protein can indicate which amino acids are likely to take direct part in protein function and which are probably not as directly involved.

Application of the second type of analysis to the available cytochrome c data shows positions of strikingly constant amino acid identity in the protein. Based on the sequences known to Dickerson in 1972 there are 35 completely invariant sites, 23 sites with variation to one other amino acid and 17 sites with variation to only two other amino acids. The remaining 28 or more positions exhibit greater variation.

Position 13 in cytochrome c is usually occupied by a lysine residue ($R = (CH_2)_4\text{-}NH_3^+$) in vertebrates and higher plants, but in certain lower plants and insects it is found to contain arginine ($R = (CH_2)_3\text{-}NH\text{-}C(NH_2)_2^+$). This is a conservative change because both lysine and arginine are basic, nitrogen-containing amino acids, and they are roughly the same size. It is conceivable that they could be independently carrying out the same function in different organisms' cytochrome c molecules.

Studies have been performed to test this hypothesis. In 1973 Margoliash and co-workers prepared cytochrome c derivatives, modified at position 13, from horse (Lys 13) and Candida krusei (Arg 13). It was shown that modified horse cytochrome c reacted at the same rate

with its reductase complex but that it had a reduced rate of electron transfer to the oxidase complex. Similarly, modified C. krusei cytochrome c was impaired in its ability to transfer electrons to oxidase but not in its rate of transfer from reductase(4). The conclusions to be drawn are that there are different sites for interaction of cytochrome c with its oxidase and reductase complexes, that position 13 is specifically involved in interaction with oxidase, and that the function of position 13 does not depend on whether it is occupied by a lysine or an arginine residue.

In this project a similar modification study was undertaken. The goal is to specifically modify arginine 13 on Saccharomyces cerevisiae (Baker's yeast) cytochrome c and ultimately to measure kinetic parameters for the electron transfer activity of the modified protein with reductase and oxidase as well as with a number of transition metal complex redox agents.

The project can be divided into three parts: purification of purchased cytochrome c, modification with phenylglyoxal of the purified protein, and separation of reaction products. The purification of S. cerevisiae cytochrome c, purchased from Sigma, was rather involved. The protein did not exhibit the expected chromatographic behavior, and it became necessary to attempt to characterize two major components. BioRex 70 cation exchange chromatography was used for separation, initially in sodium phosphate buffer (pH 7.1). Later the buffer was switched to bicarbonate (pH 7.4-7.7) because bicarbonate is used during the modification reaction.

Phenylglyoxal was chosen as the modification reagent because it reacts quickly and specifically with arginine. Cheung and Fonda have shown that the rates of reaction of phenylglyoxal with N-acetyl lysine and N-acetyl cysteine are negligable in comparison with its rate with N-acetyl arginine(8). In addition the rate of reaction between phenylglyoxal and N-acetyl arginine has been shown to be increased in bicarbonate, in comparison with several other buffers(8), indicating that bicarbonate is a good buffer in which to run the reaction.

The modification reaction involves adding two phenylglyoxal moeities per guanido group of arginine. A proposed structure for the product is shown in Figure 2 (9).

In order to increase specificity for arginine 13 modification the reaction was run under reducing conditions because arginine 13 is more accessible in reduced cytochrome c(4).

The modified product is unstable at pH 7 and room temperature, over a period of 48 hours, but stable below pH 4(9). Acetate buffer (pH 3.8) was used for storage for more than one day at 4°C.

When the reaction was run five products were found, and it became necessary to separate them in order to characterise them. Margoliash et al. were able to achieve purification of the arginine 13-modified C. krusei cytochrome c by Amberlite CG-50 cation exchange chromatography(4). It was decided to use BioRex 70 cation exchange chromatography to separate the products of modified S. cerevisiae cytochrome c.

Figure 2. Proposed reaction product of phenylglyoxal with arginine.

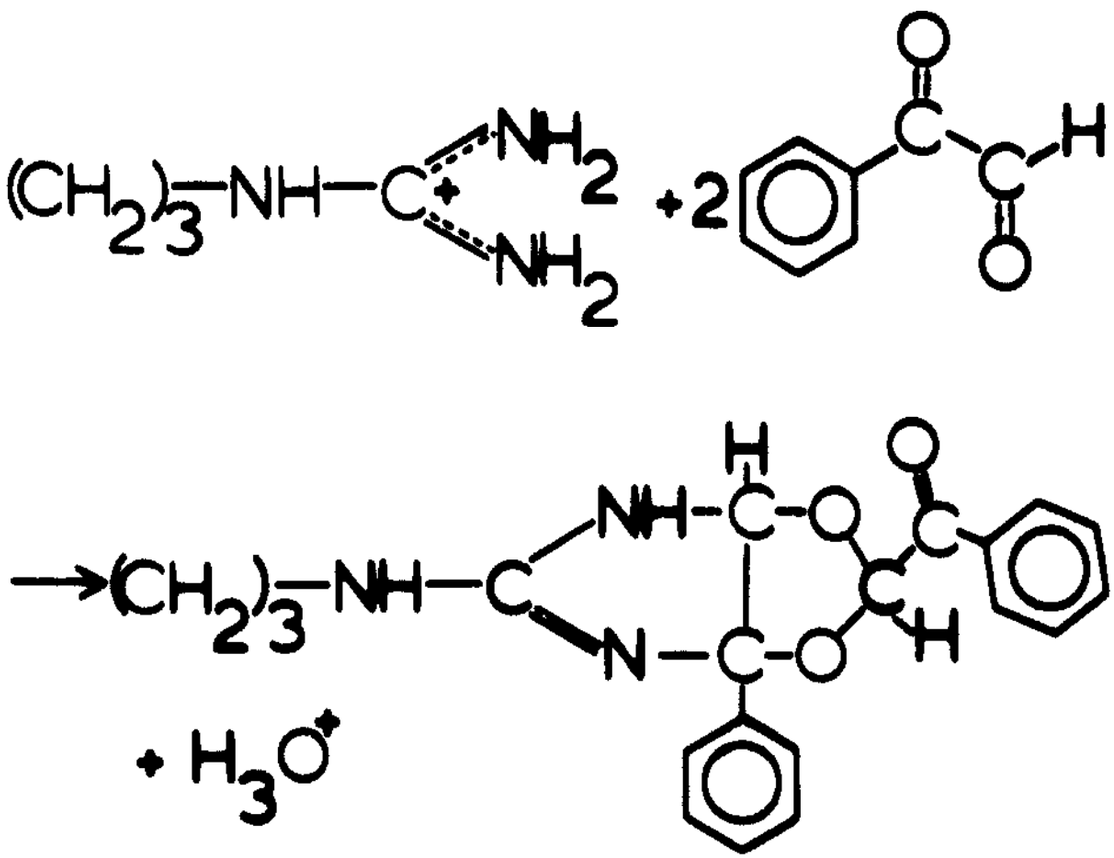

Figure 2

EXPERIMENTAL

Saccharomyces cerevisiae cytochrome c (Type VIII, lot 064F7321), phenylglyoxal monohydrate and L-ascorbic acid were purchased from Sigma Chemical Company. BioRex 70 cation exchange resin and P-6 gel were purchased from BioRad Laboratories. Amicon YM5 ultrafiltration membranes were used to concentrate protein solutions.

All buffers were prepared with degassed water that had been purified by a Sybron/Barnstead NANOpure water purification system. Sodium phosphate buffers were made from anhydrous Na_2HPO_4 purchased from J. T. Baker Chemical Company and $NaH_2PO_4 \cdot H_2O$ purchased from Mallinckrodt, Inc.

Bicarbonate buffers were made from reagent grade $NaHCO_3$ and HCl, both purchased from J. T. Baker Chemical Co. The bicarbonate was standardized against anhydrous Na_2CO_3, primary standard, purchased from Mallinckrodt, Inc. A 0.25 g sample of Na_2CO_3 was dried at 110°C for several hours, allowed to cool and accurately weighed. It was then dissolved in NANOpure water, and a few drops of methyl orange were added to the solution. This solution was titrated to the endpoint with an aqueous solution (about 0.1 M) of HCl. Before reaching the endpoint the Na_2CO_3 solution was boiled to expel the CO_2 that had formed; after cooling the solution the titration was completed. This procedure served to standardize the HCl solution.

Next, 3 ml of an aqueous solution of $NaHCO_3$ (162 g/L) were placed in a small beaker with a small amount of methyl orange. This solution was titrated to the endpoint with the standardized HCl solution, again boiling the solution to expel CO_2 when the endpoint was approached.

This procedure served to determine the concentration of the $NaHCO_3$ solution.

The titrations were always performed in triplicate, and the standardisation procedure was performed both on fresh and week-old bicarbonate solution. The results always indicated that 1.2 g/L $NaHCO_3$ solution was between 0.998 and 1.00 M. After this was determined repeated standardisations were unnecessary, and each batch of bicarbonate buffer was made from a 1 M $NaHCO_3$ solution that was prepared without having been standardised.

Acetate buffers were prepared with glacial acetic acid from Mallinckrodt, Inc. and sodium acetate from Baker and Adamson.

All pH measurements were made on a Radiometer-Copenhagen PHM84 Research pH meter. Ultraviolet/visible spectra were made on a Varian Cary 219 spectrophotometer. Chromatographic elution profiles were made at 4°C, using a LKB 2132 pump, an Isco Column Monitor, and a LKB 2070 Ultra Rac II. A Wilkens Anderson Company #2314 centrifuge was used to separate precipitate from solution in the reaction product mixtures. HPLC was performed using a Beckman Model 332 Gradient Liquid Chromatograph and an Altex Ultrapore RPS (C_3) column (4.6 mm ID x 7.5 cm).

Amino acid analysis was performed by the University of Illinois Biotechnology Center, using a PICO-TAG Work Station from Waters Associates. The sample was prepared for amino acid analysis by ultrafiltration into degassed, unbuffered MANOpure water.

Initial BioRex 70 chromatography of cytochrome g was in sodium phosphate buffer (pH 7.1). The ionic strength was varied between

u = .35 M and u = 1.0 M until u = .65 M was found to be practical. High ionic strength was required to cause the more tightly bound component to move. The sodium phosphate columns were run by hydrostatic pressure at room temperature because refrigeration sometimes caused the buffer to precipitate in the tubing. Because of the precipitation problem no elution profile was recorded for phosphate column runs. The flow rate was always between 20 and 25 ml/hr (except during loading when it was 10-15 ml/hr). Potassium ferricyanide was added to all samples before loading. Column dimensions were 1.4 x 16 cm.

BioRex 70 chromatography was also performed with bicarbonate buffer (pH 7.4-7.7). Even when kept at 4°C the pH of these buffers increased slowly with time due to loss of CO_2. All bicarbonate columns were run at 4°C because spontaneous formation of CO_2 bubbles in the tubing occured at room temperature. An ionic strength of u = .35 M was found to be practical for separation of the two components. When the elution was monitored the flow rate was 5 ml/hr. When run by hydrostatic pressure, flow rate was about 15 ml/hr. Potassium ferricyanide was added to all samples before loading. Column dimensions were 1.4 x 16 cm. Sample sizes ranged from 8 to 50 mg, and each could be cleanly separated into the two components.

The two components of Sigma's cytochrome c will be referred to as Band I and Band II according to the order in which they elute from the BioRex column (Band I elutes first). The modification reaction was run on Band II. The collected Band II was ultrafiltered over a YM5

membrane and diluted with u = .08 M bicarbonate in order to reduce the ionic strength to about 0.1 M. The pH was measured and usually found to be about 8.3; it was then adjusted to 7.6 with 1.0 M HCl. Sample sizes between 7.5 and 30 mg were used. The phenylglyoxal was added, in forty molar excess, as a 2 ml solution in .08 M bicarbonate, over a 48 hour period. Usually ten drops of solution were added at six to ten hour intervals.

The cytochrome c solution was put into a serum bottle with a stir bar and deaerated by bubbling argon through it. A minimal amount of L-ascorbic acid was added to reduce the cytochrome c; reduction was accompanied by a noticable color change. The first batch of phenylglyoxal solution was added, and the reaction was allowed to run under argon, with stirring.

All reaction mixtures were ultrafiltered immediately after removing them from the argon atmosphere, in order to rid them of excess phenylglyoxal. Some were ultrafiltered into acetate buffer (u = .08 M, pH 3.8) and stored at 4°C; those that were not stored in acetate were chromatographed immediately following ultrafiltration. Reverse phase high performance liquid chromatography (HPLC) was performed on a reaction mixture in acetate in order to determine how many reaction products had formed. Samples were pre-filtered to remove any particulate matter, and they were run with the generous assistance of James Schwartz. The gradient used for elution was 0-50% B in 45 minutes where A was 0.1% trifluoric acetic acid (TFA) in water and B was 90% acetonitrile, 0.1% TFA and 9.9% water.

P-6 gel filtration was performed in acetate buffer. The filtration used to prepare the reaction mixture for HPLC analysis was run at room temperature by hydrostatic pressure. Other filtrations were run at 4°C with the pump set at 2 ml/hr. Column dimensions were 1.5 x 21.5 cm.

A 1.4 x 16 cm BioRex 70 column was used to attempt to separate reaction products. These elutions were run using either bicarbonate buffer (pH 7.4-7.7) or degassed, unbuffered NANOpure water (pH 5.6). Reaction mixtures were ultrafiltered into the appropriate medium; they were concentrated to a volume (2-10 ml) that was convenient for loading. Columns were run at 4°C, at 5 ml/hr.

RESULTS AND DISCUSSION

PURIFICATION

Sigma's yeast cytochrome c separates cleanly into two major components on BioRex 70 under the specified conditions. Visible spectra of the two components in sodium phosphate buffer (Figure 3) indicate that there was 61% Band II and 39% Band I in the original sample. It should be noted that these numbers assume fully oxidised Band I and Band II, yet Band II appears to be slightly reduced. These numbers are consistent with those obtained for separation in bicarbonate buffer, when the elution profile was recorded (Figure 4). "Band I" eluted as four peaks. Visible spectra of the five heme-containing peaks from Figure 4b (Figure 5) indicate 65% Band II and 35% Band I. Again, these numbers assume fully oxidised Band I and Band II whereas Band II and one peak from Band I appear to be somewhat reduced.

The identities of the two major components have not yet been determined. In 1965 Slonimski et al. reported the presence of two distinct species of cytochrome c in yeast, separable by Amberlite XE64 cation exchange chromatography(5). Iso-1 and iso-2, as they were called, comprised about 80% and 20%, respectively, of the wild-type cytochrome c. It was thought that the two bands might be iso-1 and iso-2.

The two isozymes are quite similar in their physical properties. E = +247 mV, extinction coefficient(550 nm) = 28 $mM^{-1}cm^{-1}$, molecular weight = 12,500(iso-1) and 12,800(iso-2) (5). Several characteristic differences do exist, though.

Figure 3. Visible spectra of Band I (broken lines) and Band II (solid lines) of yeast cytochrome c in sodium phosphate buffer (pH 7.2). Band I was in u = 0.50 M phosphate, and the same buffer was used in the reference cell. Band II was in u = 1.0 M phosphate, and the same buffer was used in the reference cell. Absorbance range = 1.0 for the Soret maxima at 410 nm. The maxima at 550 nm were also recorded at absorbance range = 0.10 (Band I) and absorbance range = 0.20 (Band II). Percentage calculations were based on an extinction coefficient of 105 $mM^{-1}cm^{-1}$ for the Soret maximum of oxidised cytochrome c.

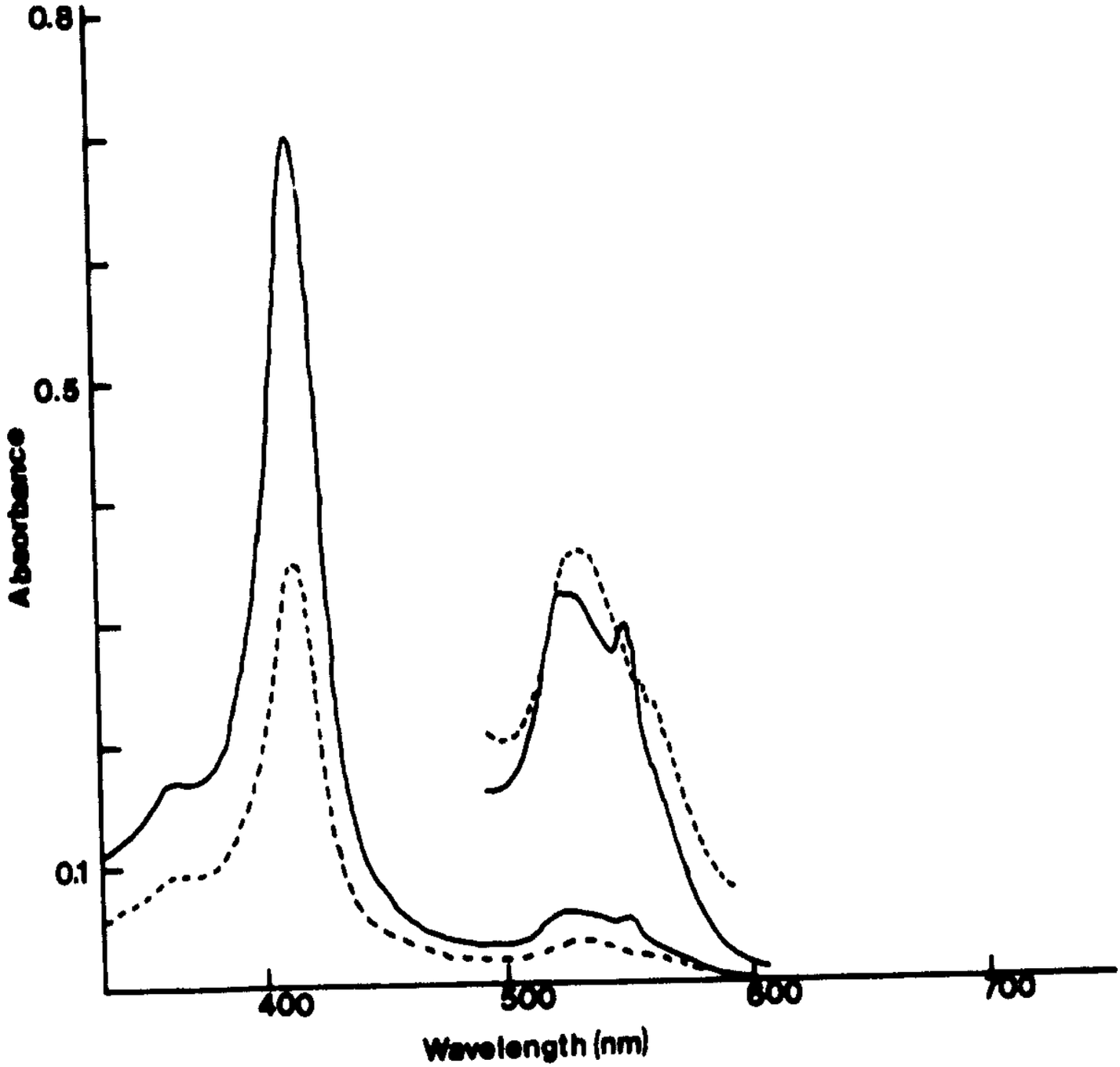

Figure 3

Figure 4. Elution profiles, recorded at 405 nm, of Sigma's yeast cytochrome c in bicarbonate buffer. The peak at 21 ml, in both figures 4a and 4b, was caused by ferricyanide. 4a) A 10.0 mg cytochrome c sample was loaded. The run was started with bicarbonate (pH 7.7) having ionic strength less than 0.5 M, but at the arrow (60 ml) the ionic strength was increased to 0.5 M in order to elute Band II 4b) An 8.9 mg sample of cytochrome c was loaded. The run was started with bicarbonate (pH 7.6) having ionic strength of 0.30 M, but at the arrow (195 ml) the ionic strength was increased to 0.50 M in order to elute Band II.

Figure 4a

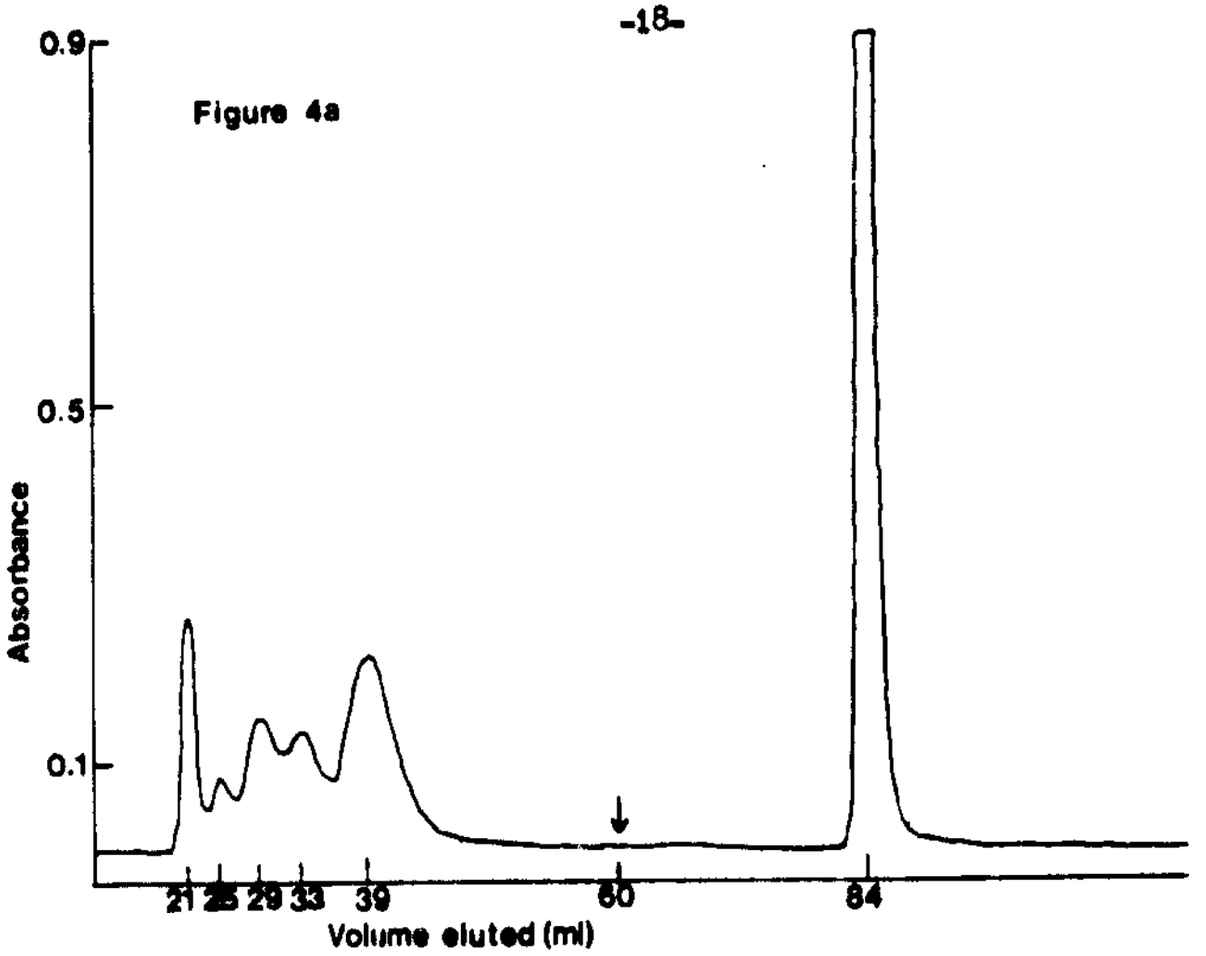

Figure 4b

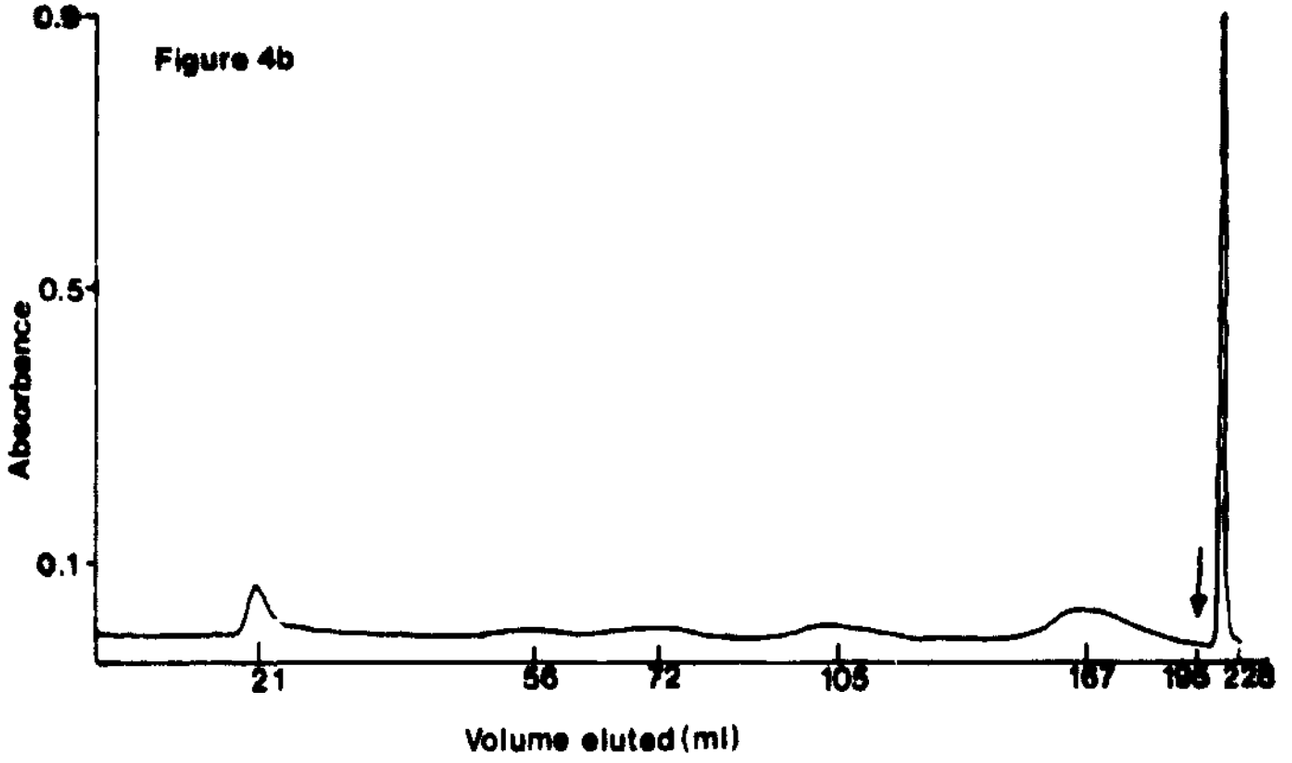

Figure 5. Visible spectra of the five heme-containing peaks from Figure 4b: 51-60 ml, 63-78 ml, 90-120 ml, 150-189 ml and 216-228 ml. Bicarbonate buffer (u = .08 M, pH 7.6) was used in the reference cell. Band II (216-228 ml) is shown as broken lines, and Band I peaks are shown as solid lines. The smallest absorbance is for 51-60 ml, the next smallest for 63-78 ml, the next for 90-120 ml, and the largest Band I peak is for 150-189 ml. Absorbance range = 0.5 was used for all spectra, but the Band II Soret maximum required a Suppression setting of 2.0. Percentage calculations were based on an extinction coefficient of 105 $mM^{-1}cm^{-1}$ for oxidised cytochrome c.

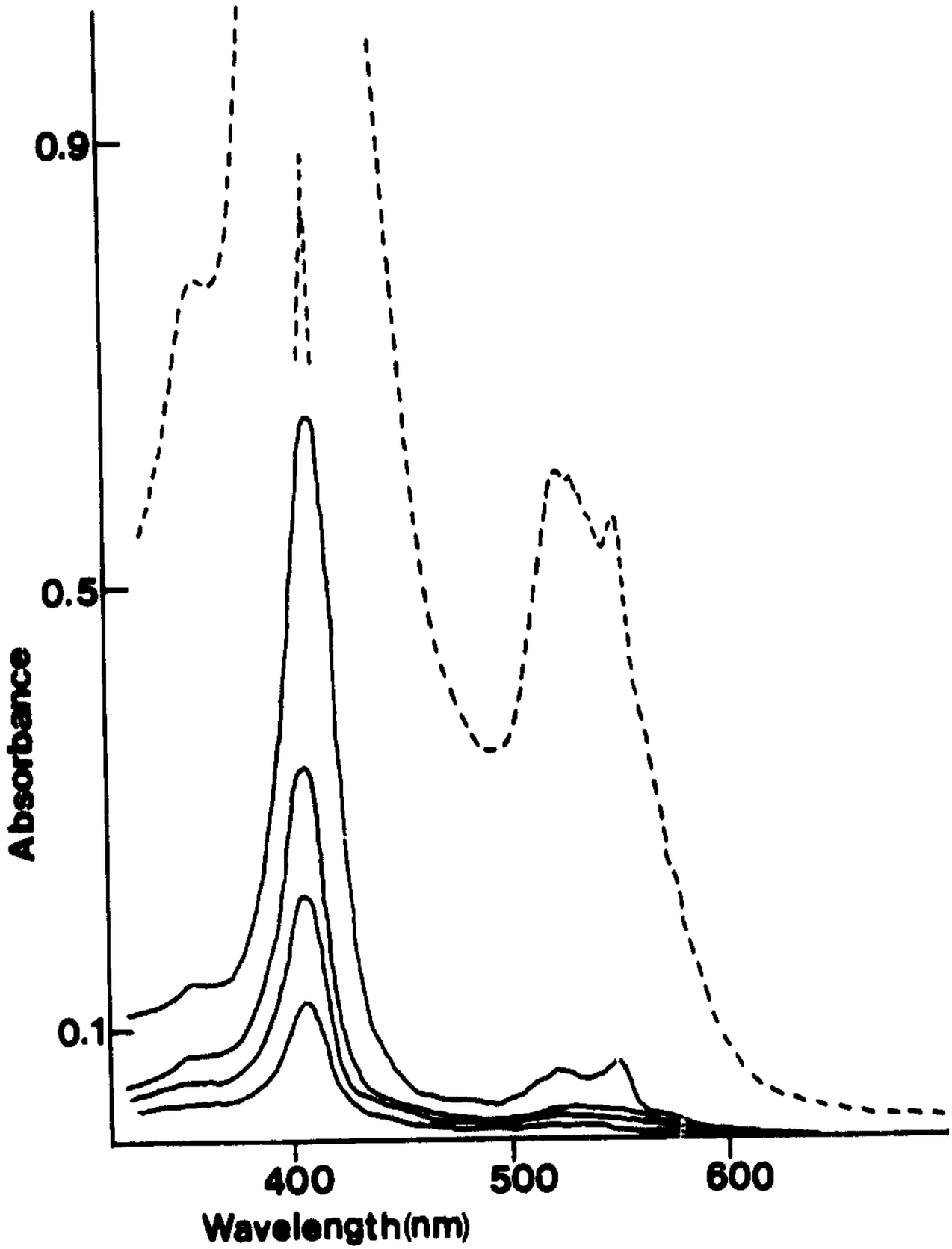

Figure 5

Both complete amino acid sequences are known(6), and there are amino acid differences at eighteen of the positions from 1 to 103. In addition iso-2 has an extended amino terminus of four amino acid residues. Both of the amino termini are methionine residues, but iso-2 has lysine at its carboxy terminus whereas glutamic acid occupies that site in iso-1. The calculated charge at pH 7 is 7½ for iso-2 and 7 for iso-1. Iso-2 contains only two cysteine residues which are coordinated to the heme, but iso-1 contains, in addition to these two, an uncoordinated cysteine at position 102; this should result in a difference in free sulfhydryl reactivity between them(7). Spectral shifts are small, but it is possible to distinguish between the two cytochromes by difference spectroscopy at 550 nm(5).

The charge difference suggests that iso-1 may elute from BioRex 70 before iso-2 and thus that Band II is iso-2; however, this is not conclusive. The differences in sulfhydryl content, carboxy terminus and 550 nm absorption position have not yet been exploited in order to identify the two components. Band II was submitted for amino acid analysis, and the results were compared to calculated percent compositions for iso-1 and iso-2 (Table 1). In doing the calculations the tryptophan in each sequence was omitted because acid hydrolysis of peptide bonds degrades tryptophan. Also the two cysteines covalently bound to the heme were omitted.

There are several points to be made in comparing the numbers. First, the large discrepancies between experimental and calculated values for ASX, GLX and LYS also appeared (and in the same direction)

Table 1. Amino acid analysis of Band II(a) compared with calculated values for iso-1 and iso-2. Amino acid analysis of purified horse heart cytochrome c(b) compared with calculated values. In doing the calculations the tryptophan in each sequence was omitted because acid hydrolysis of peptide bonds degrades tryptophan. Also the two cysteines covalently bound to the heme were omitted. Analyses were performed by the University of Illinois Biotechnology Center.

	ISO-1	ISO-2	FOUND
ASX	10.5	10.9	14.2
GLX	8.6	8.2	10.4
SER	3.8	5.4	3.4
GLY	11.4	10.9	12.1
HIS	3.8	2.7	3.3
ARG	2.8	2.7	7.6
THR	7.6	8.2	2.9
ALA	6.7	7.3	8.2
PRO	3.8	4.5	4.5
TYR	4.8	4.5	3.8
VAL	2.8	2.7	2.8
MET	2.8	3.6	1.4
CYS	.95	---	---
ILE	3.8	4.5	2.3
LEU	7.6	4.5	6.9
PHE	3.8	3.6	3.6
LYS	15.2	15.4	12.4

Table 1a

	HORSE	FOUND
ASX	7.9	10.8
GLX	11.9	15.8
SER	---	---
GLY	13.6	11.9
HIS	3.2	3.0
ARG	10.0	2.0
THR	2.2	9.9
ALA	6.5	6.0
PRO	4.0	3.0
TYR	3.9	4.0
VAL	3.2	3.0
MET	1.8	2.0
CYS	---	---
ILE	4.7	6.0
LEU	6.0	5.9
PHE	3.6	5.0
LYS	10.6	18.8

Table 1b

when purified horse heart cytochrome g was submitted by Jean Kula for analysis (Table 1). Second, arginine and threonine have similar retention times, and it is likely that the experimental values obtained for them should be reversed. Certainly the numbers obtained indicate that this needs to be done, and a similar discrepancy appeared in the analysis of purified horse heart cytochrome g. Third, the two most striking differences between iso-1 and iso-2 amino acid composition lie in their leucine and serine contents; both of these numbers indicate that Band II is iso-1.

Charge and amino acid composition give conflicting identifications of Band II. Charge indicates that Band II is iso-2, and amino acid content indicates that it is iso-1. The amino acid analysis is more reliable than the prediction based on charge, however. Although the identification is not definite it was decided to run the modification reaction on Band II. When compared with the discrepancies apparent in the horse cytochrome g analysis, the numbers obtained for yeast cytochrome g, Band II, are consistent with its being either iso-1 or iso-2. Band II does not show the same inhomogeneity in BioRex 70 elution as Band I (Figure 4), and there is more Band II in Sigma's product. Finally, both isozymes should be susceptible to the desired modification reaction because both contain arginine at position 13.

MODIFICATION

The modification reaction was run on Band II, and gel filtration in acetate was performed on the reaction products of 10 mg cytochrome g with phenylglyoxal. The elution profile (Figure 6) shows a single, slightly asymmetric peak, indicating that no fragmentation had occured during the reaction.

Figure 6. Elution profile, monitored at 405 nm, of a mixture of products from the reaction of 10 mg yeast cytochrome *c*, Band II, with a forty molar excess of phenylglyoxal. The mixture was eluted through P-6 gel at 2 ml/hr, at 4°C.

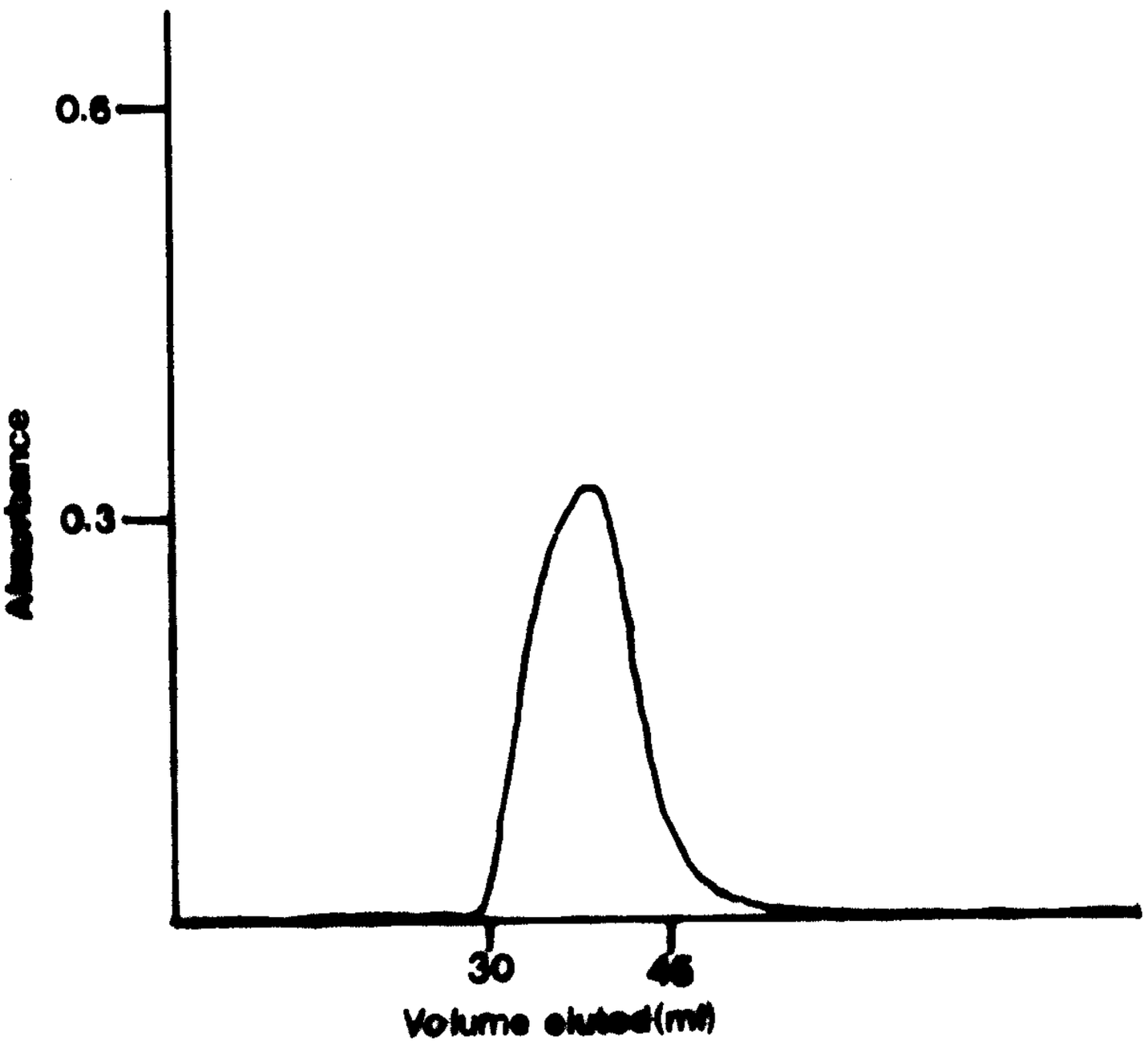

Figure 6

Gel filtration was performed on this reaction mixture after removing by centrifugation a red precipitate that had formed. This precipitate appeared to have formed during the time when the mixture was under reacting conditions because the mixture that was ultrafiltered was cloudy. However, the precipitate did not collect at the bottom of the storage tube until left in the refrigerator for a couple of days. Vigorous bubbling of argon through the reacting mixture seemed to increase the amount of precipitate that eventually formed, but a small amount of precipitate formed even when treatment with argon was gentle.

HPLC, under the specified conditions, was unable to distinguish between Band I and Band II of Sigma's yeast cytochrome c. Unpurified yeast cytochrome c in acetate showed one major peak with a retention time of 34.2 minutes and two minor peaks with retention times of 33.2 and 32.8 minutes (Figure 7). Purified Band II in bicarbonate showed one major peak at 34.0 minutes and several tiny peaks at 33.4, 32.8, 32.3 and 30.1 minutes (Figure 8). Phenylglyoxal in bicarbonate eluted completely in the void volume of the column. The gel filtered mixture of Band II reacti n products in acetate showed five peaks in addition to unreacted cytochr me c at 34.3 minutes (Figure 9). These peaks had retention times of 37.6, 38.2, 39.2, 41.1 42.4 minutes, with the largest of these appearing at 39.2 minutes.

These five peaks were believed to be arginine-modified cytochrome c. Three arginines appear in each of iso-1 and iso-2, at positions 13, 38 and 91. The three least hydrophobic of the five peaks, then, are

Figure 7. HPLC chromatogram of unpurified yeast cytochrome c (Sigma, Type VIII, lot 064F7321). The gradient used was 0-50% B in 45 minutes where A was 0.1% TFA in water and B was 90% acetonitrile, 0.1% TFA and 9.9% water. The retention time of the major peak was 34.2 minutes. The retention times of the two minor peaks were 33.2 and 32.8 minutes. The early peaks were caused by solvent molecules; the cytochrome c had been dissolved in acetate buffer.

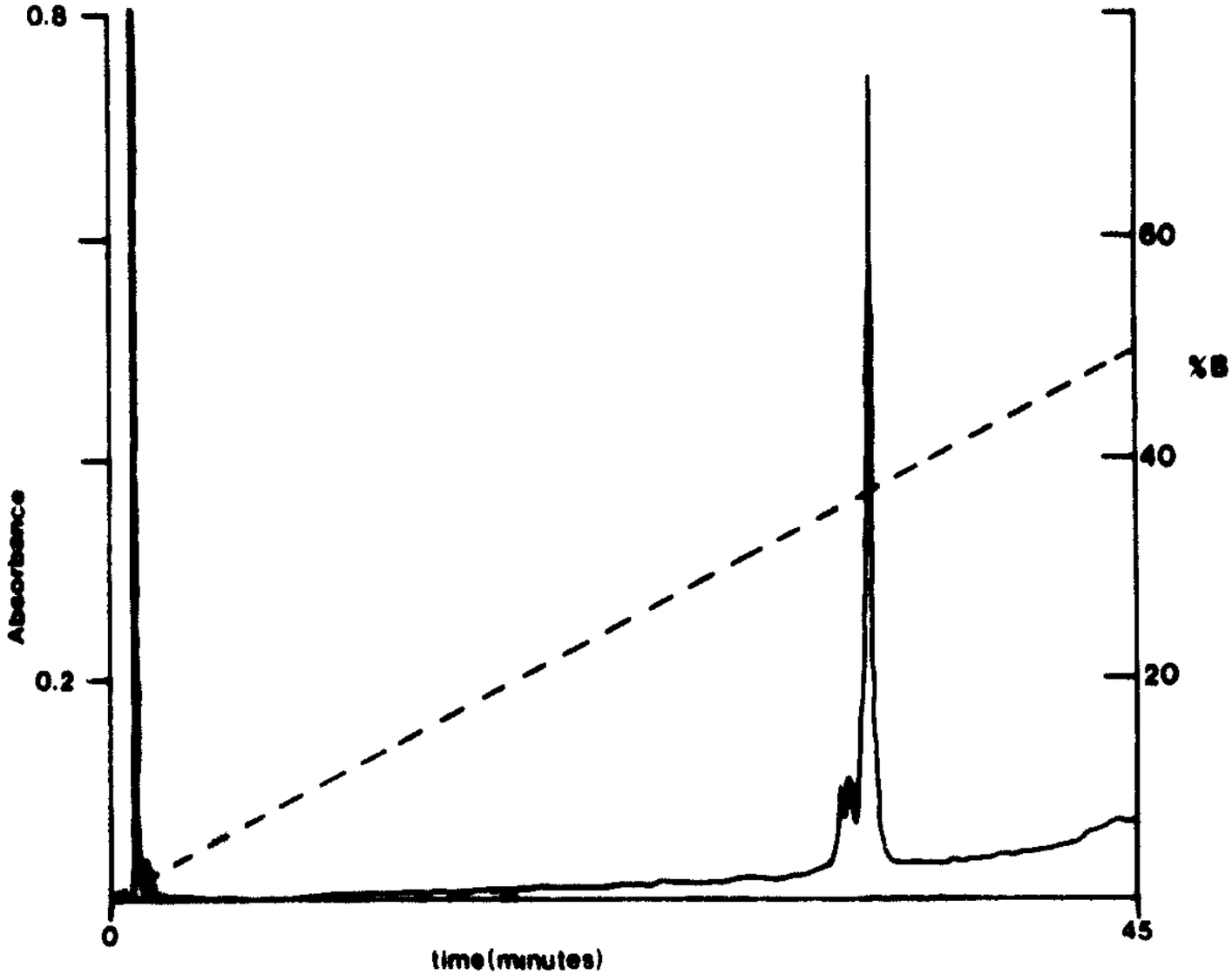

Figure 7

Figure 8. HPLC chromatogram of purified Band II of yeast cytochrome c. The gradient used was 0-50% B in 45 minutes where A was 0.1% TFA in water and B was 90% acetonitrile, 0.1% TFA and 9.9% water. The retention time of the major peak was 34.0 minutes. The retention times of the four nearby tiny peaks were 33.4, 32.8, 32.3 and 30.1 minutes. The early peaks were caused by solvent molecules; the Band II cytochrome c had been dissolved in bicarbonate buffer.

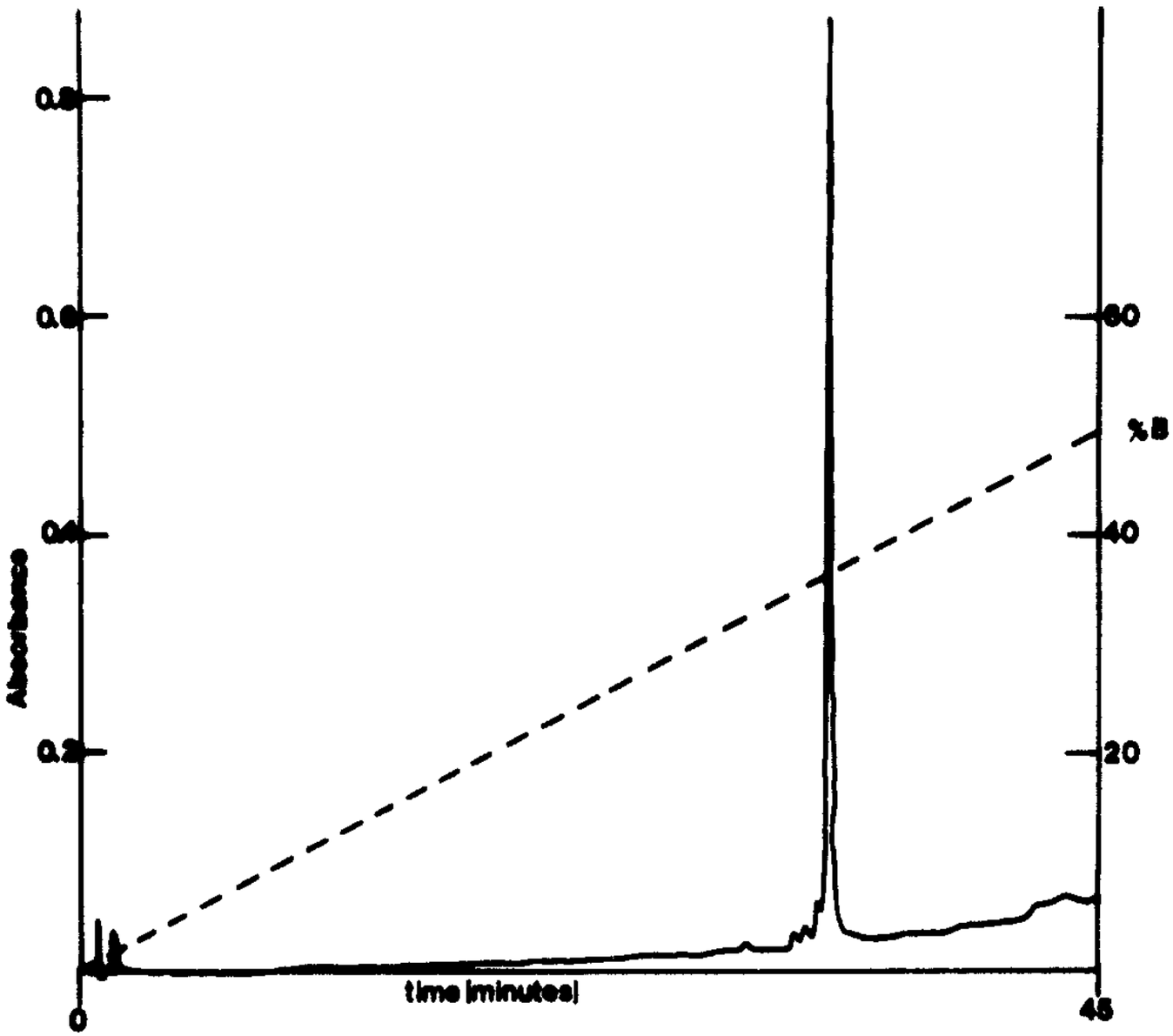

Figure 8

Figure 9. HPLC chromatogram of the products of reaction of purified Band II of yeast cytochrome g with phenylglyoxal. The reaction mixture had been ultrafiltered and gel filtered prior to the HPLC run. The gradient used was 0-50% B in 45 minutes where A was 0.1% TFA in water and B was 90% acetonitrile, 0.1% TFA and 9.9% water. The retention times of unreacted cytochrome g and five reaction products are indicated in the figure. The early peaks were caused by solvent molecules; the reaction mixture had been dissolved in acetate buffer.

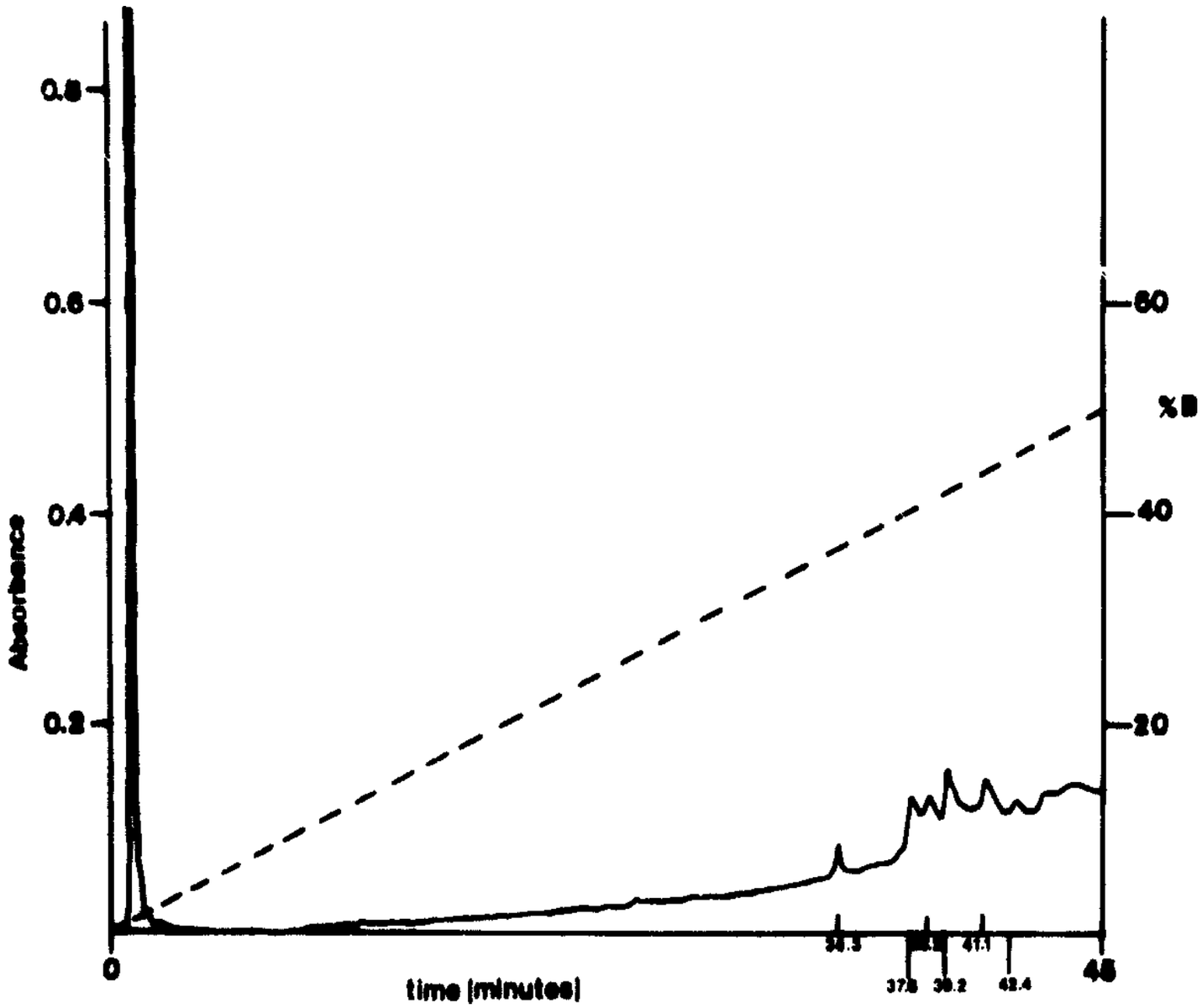
0.8
0.6
0.4
0.2
Absorbance
60
%B
40
20
0
time (minutes)
37.8
39.2
41.1
42.4
45

Figure 9

probably singly modified species, and the peaks at 41.1 and 42.4 minutes may be doubly modified. It is hoped that the largest peak, with a retention time of 39.2 minutes, is arginine 13-modified, but that has yet to be shown. Margoliash reports, in his work with *C. krusei* modifications, that the main modified product of a reaction run under similar conditions was specifically modified at position 13(4).

SEPARATION

The products of reaction all eluted together as a single peak from BioRex 70 in 0.50 M bicarbonate, 0.08 M bicarbonate and pure water, with an elution volume each time of about 20 ml. The general shape of the peak is shown in Figure 10; this profile was recorded for the run in pure water.

When the column was run at low ionic strength (0.08 M bicarbonate or pure water) some heme-containing species remained at the top of the column. It was assumed to consist of unreacted cytochrome *g*. The assumption is supported by the fact that when the peak in Figure 10 was reloaded onto the same column, less heme eluted in the 20 ml volume and more was stuck at the top of the column. A degradation phenomenon could be the result of excessive ultrafiltration or of the expected instability of phenylglyoxal modified arginine residues above pH 4, or it could be a combination of the two effects. In any case, disappointing resolution of the reaction products was obtained. Perhaps an attempted separation on the Amberlite resin used by Margoliash would yield more satisfactory results.

Figure 10. BioRex 70 elution profile of the products of a modification reaction between purified Band II and phenylglyoxal. This elution was run in degassed, unbuffered NANOpure water at 5 ml/hr, at $4^{o}C$.

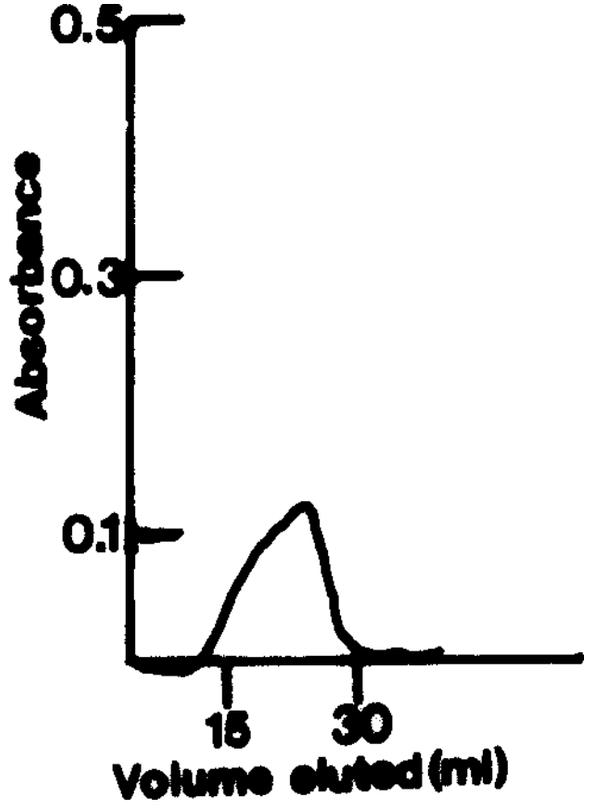

Figure 10

It is also possible that the reaction products could be separated by anion exchange chromatography; they do seem to run through BioRex 70 in the void volume. However, this would be a surprising result because it is unlikely that reaction with phenylglyoxal could convert such a positively charged species (+7 or +7½) to an anion.

CONCLUSION

The specific modification of arginine 13 on *Saccharomyces cerevisiae* cytochrome *c* is desired in order to determine the effect on the kinetic parameters of the modified protein with reductase and oxidase complexes.

A separation scheme was devised in order to purify each of two components of the yeast cytochrome *c* purchased from Sigma. BioRex 70 chromatography in $u = 0.35$ M bicarbonate buffer, at $4^{\circ}C$, cleanly separated the two components.

The identities of the two components have not yet been definitely assigned. Based on the results of amino acid analysis, it seems probable that Band II (which elutes after Band I) is the iso-1 form of yeast cytochrome *c*.

The modification reaction with phenylglyoxal was run on Band II. The resulting reaction mixture was characterised by reverse phase HPLC, and five reaction products were found. Separation of the reaction products by BioRex 70 chromatography was attempted, but resolution into individual products was not achieved. Perhaps either Amberlite CG-50 or some form of anion exchange chromatography would improve the separation.

REFERENCES

(1) Dickerson, R. (1972) Scientific American 226(4), pp. 58-72.

(2) Stryer, L. (1981) Biochemistry(W. H. Freeman, Inc., San Francisco), p. 328.

(3) Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. (1983) Molecular Biology of the Cell(Garland Publishing Co., New York), p. 507.

(4) Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C., Feinberg, B., Brautigan, D. and Morrison, M. (1973) Proc. Natl. Acad. Sci. 70(11), pp. 3245-3249.

(5) Slonimski, P., Acher, R., Pere, G., Sels, A. and Soulo, M. (1965) in Regulations Chez les Micro-organismes(Centre Nationale de la Recherche Scientifique, Paris), pp. 435-461.

(6) Montgomery, D., Leung, D., Smith, M., Shalit, P., Faye, G. and Hall, B. (1979) Proc. Natl. Acad. Sci. 77(1), pp. 541-545.

(7) Ayrovainen, M., personal communication.

(8) Cheung, S. and Fonda, M. (1979) Biochem. Biophys. Res. Comm. 90(3), pp. 940-947.
see also Lundblad, R. and Noyes, C. (1984) Chemical Reagents for Protein Modification, v. 2(CRC Press, Inc., Boca Raton, Florida), pp. 1-45.

(9) Takahashi, K. (1968) J. Biol. Chem. 90(3), pp. 6171-6179.
see also Glaser, A. (1970) Ann. Rev. Biochem. 39, p. 101.