Differential Labeling of the Subunits of Respiratory Complex III with [³H]Succinic Anhydride, [¹⁴C]Succinic Anhydride, and *p*-Diazobenzene-[³⁵S]sulfonate

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Abstract

Exposure of antimycin-treated Complex III (ubiquinol-cytochrome c reductase) purified from bovine heart mitochondria to [³H]succinic anhydride plus [³⁵S]p-diazobenzenesulfonate (DABS) resulted in somewhat uniform relative labeling of the eight measured subunits of the complex by [³H]succinic anhydride. In contrast, relative labeling by [³⁵S]DABS was similar to [³H]succinic anhydride for the subunits of high molecular mass, i.e., core proteins, cytochromes, and the iron–sulfur protein, but greatly reduced for the polypeptides of molecular mass below 15 kDa. With Complex III depleted in the iron–sulfur protein the relative labeling of core protein I by exposure of the complex to [³H]succinic anhydride was significantly enhanced, whereas labeling of the polypeptides represented by SDS-PAGE bands 7 and 8 was significantly inhibited. Dual labeling of the subunits of Complex III by ¹⁴C- and ³H-labeled succinic anhydride before and after dissociation of the complex by sodium dodecyl sulfate, respectively, was measured with the complex in its oxidized, reduced, and antimycin-inhibited states. Subunits observed to be most accessible or reactive to succinic anhydride were core protein II, the iron–sulfur protein, and polypeptides of SDS-PAGE bands 7, 8, and 9. Two additional polypeptides of molecular masses 23 and 12 kDa, not normally resolved by gel-electrophoresis, were detected. Reduction of the complex resulted in a significant change of $^{14}C/^3H$ labeling ratio of core protein only, whereas treatment of the complex with antimycin resulted in decreases in $^{14}C/^3H$ labeling ratios of core proteins I and II, cytochrome c_1, and a polypeptide of molecular mass 13 kDa identified as an antimycin-binding protein.

Key Words: Ubiquinol-cytochrome c reductase; Complex III; subunits; polyacrylamide gel electrophoresis; diffential labeling; succinic anhydride; *p*-diazobenzenesulfonate.

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Introduction

Considerable evidence suggests that conformational transitions play a vital role in the function of mitochondrial ubiquinol-cytochrome c reductase (Complex III). The earliest evidence was the observation that the susceptibility of the complex to cleavage by bile salts plus ammonium sulfate or guanidinium salts was highly dependent on the redox state of the complex (Rieske et al., 1967). Subsequent studies have reported other phenomena that can best be attributed to conformational transitions. These include redox-linked alterations in liposomal paracrystalline arrays (Wakabayashi et al., 1972), circular dichroic spectra (Reed et al., 1978, 1979), buried sulfhydryl groups as detected by infrared spectroscopy (Rieske et al., 1975), and bound maleimido-linked nitroxide groups used as a spin label (Das Gupta et al., 1979). However, except for the circular dichroic measurements applied to heme prosthetic groups of cytochromes b and c_1, these measurements could be applied only to the overall complex, thus giving little information concerning contributions to overall conformational transitions of individual polypeptide subunits. Even spectroscopic measurements involving individual subunits possessing redox prosthetic groups cannot yield information concerning conformational changes involving subunits possessing no detectable prosthetic groups.

Okunuki and co-workers (Nozaki et al., 1958; Yamanaka et al., 1959) first reported that the accessibility of cytochrome c to protein-modifying reagents such as bacterial protease or monoiodoacetic acid varied with the oxidation state of the cytochrome. These observations together with the availability of radiolabeled alkylating reagents and polyacrylamide electrophoretic procedures for separation of the polypeptide subunits of Complex III suggested the use of these techniques for the measurement of contributions of individual subunits of the complex toward conformational transitions observed with different redox or inhibited states of the complex.

Considerable use has been made of protein-modifying reagents as probes of the relative accessibilities of the polypeptide subunits of Complex III and thus a possible measure of the topological relationship among the subunits. These probes have included ^{125}I plus lactoperoxidase (Gellerfors and Nelson, 1977; D'Souza and Wilson, 1982), ^{125}I plus chloramine T (Gellerfors and Nelson, 1977), p-diazobenzene[^{35}S]sulfonate (DABS)[4] (Gellerfors and Nelson, 1977; Mendel-Hartvig and Nelson, 1978; Bell et al., 1979), and proteases such as trypsin or chymotrypsin (Trumpower and

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[4]Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; antimycin, antimycin A; DABS, p-diazobenzenesulfonate; DTE, dithioerythritol; DMSO, dimethylsulfoxide.

Katki, 1975; Ball *et al.*, 1977; Sidhu and Beattie, 1982; Mendel-Hartvig and Nelson, 1983). In this study, we have investigated the application of differential and dual labeling by *p*-diazobenzene-[^{35}S]sulfonate ([^{35}S]DABS), [^{3}H]succinic anhydride, and [^{14}C]succinic anhydride to the detection of possible structural and conformational changes in subunits of Complex III associated with removal of the iron–sulfur protein, change in redox state, and binding of antimycin.

Materials and Methods

Complex III was purified from bovine-heart mitochondria by the procedure described by Rieske (1967). The enzyme was freed of Tris and ammonium ions by precipitation and sedimentation in 48% ammonium sulfate followed by solution of the pellet in a phosphate buffer (0.05 M Na$_2$HPO$_3$, pH 7.5), 0.67 M sucrose, and dialysis against this buffer–sucrose mixture for 3 hr to remove residual ammonium sulfate. Finally, the turbid suspension was centrifuged 30 min at 30,000 rpm, the red pellet suspended in the phosphate–sucrose mixture, and solubilized by addition of potassium deoxycholate to 0.5 mg per mg protein. [^{3}H]succinic anhydride (23.8 Ci/mmole) was custom-synthesized (New England Nuclear Co.) by the catalytic reduction of maleic anhydride with carrier-free tritium gas. [^{14}C]succinic anhydride (20 mCi/mmole) also was obtained from New England Nuclear Co. [^{35}S]sulfanilic acid (103 mCi/mmole) was obtained from Amersham Co. Antimycin A was a gift from Ayerst Laboratories, Montreal, Canada through the courtesy of Dr. Claude Vezina. Sodium dodecyl sulfate (SDS) was purchased from BDH through Gallard-Schleshinger Chemical Co. Acrylamide and methyl-bisacrylamide were obtained commercially and recrystallized from acetone before use. Sodium taurocholate was obtained from Calbiochem and was recrystallized first from aqueous sodium chloride then from ethanol–ether. All other chemicals were of highest purity available commercially and were used without further purification.

Electrophoretic Procedures

SDS-PAGE employed in this study was a modification of the procedures of Furthmayr and Timpl (1971) and Weber and Osborn (1969) emphasizing the counterion effect (Rodbard and Chrambach, 1971) as described in a previous report (Ho *et al.*, 1985).

Preparation of p-Diazobenzene-[^{35}S]sulfonate ([^{35}S]DABS)

A procedure modified from the procedure of DePierre and Karnovsky (1974) was used. Sulfanilic acid (13 mg, 103 mCi/mmol ^{35}S) was dissolved in

0.68 ml of 0.37 M KCl containing 8.44 mg $NaNO_2$ and maintained at 0°C in ice. HCl (30 μl, 12 N) was added with stirring after which the mixture was allowed to stand in ice for 20 min. After crystallization of p-diazobenzene-sulfonate the excess nitrous acid was decomposed by addition of 5.4 mg sulfamic acid. After standing overnight the solution was free of nitrous acid as indicated by starch iodide test paper. The crystalline product was collected by sedimentation and dissolved in 0.34 ml of 0.37 M KCl acidified by 30 μl 12 N HCl. The yield of [^{35}S]DABS was 62% of theoretical. The stock solution (124 mM in [^{35}S]DABS was divided into three equal aliquots and stored in the dark at -20°C.

Labeling of Complex III

Complex III (in the ammonium/Tris-free buffer) was labeled by a 30-min exposure at 0°C of 0.05–0.2 ml containing 1.5–4 mg protein to amounts of labeling reagents that yielded substoichiometric incorporation (with respect to the cytochrome c_1 content of Complex III) of the labeling reagents. After exposure to DABS, unreacted DABS was decomposed by addition of sodium dithionite. The protein then was precipitated by 90% acetone. The precipitated protein was sedimented, rinsed with water, and then dissolved in a dissociating buffer consisting of 0.1 M sodium phosphate, pH 7.0, 10% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol or sucrose (w/v), and 1.0% (v/v) mercaptoethanol.

Other Procedures

Protein was determined by a biuret procedure or by a modified Lowry procedure with bovine serum albumin as a standard. Gels were scanned at 600 nm in a Gilford Model 2000 spectrophotometer equipped with a gel scan attachment. Gel slices (1 mm) were digested in 0.3 ml 30% H_2O_2 at 80°C for 3 hr or at room temperature for 3 days after which an aqueous counting scintillant was added. Standard curves for ^{35}S, ^{3}H, and ^{14}C were made by plotting the counting efficiency vs. channel ratio or automatic internal standards in a series of quenched standards. Counting was accomplished with a Beckman Model S7000 liquid scintillation counter. Dual isotope counting utilized Program No. 6.

Results

Dual Labeling of Subunits of Complex III by [^{3}H]Succinic Anhydride and [^{35}S]DABS

p-Diazobenzenesulfonate (DABS) has found considerable use as a labeling probe of surfaces of membrane-bound proteins and multisubunit enzyme

complexes. The charged diazo and sulfonate groups purportedly hinder penetration of the compound beyond reactive groups on the surfaces of native proteins. In contrast, succinic anhydride is a small uncharged molecule and thus would be expected to penetrate to a greater extent the tertiary and quaternary interstices of a multisubunit complex such as Complex III. To test the relative accessibility of individual subunits of isolated Complex III to these labeling reagents, a dual isotope-labeling experiment using [^{3}H] succinic anhydride and [^{35}S]DABS was performed and the distribution of the isotopes among the polypeptide subunits of Complex III was measured. Under the conditions used, less than one molecule of either [^{3}H]succinic anhydride or [^{35}S]DABS per molecule of Complex III was incorporated. In all of the labeling experiments conducted in this study substoichiometric incorporation of the label was maintained to minimize secondary labeling subsequent to possible conformation modifications arising from the primary labeling.

The results as shown in Fig. 1 demonstrate that the subunits of Complex III differ greatly in their accessibility or reactivity to the two probes. PAGE bands 1, 3, 4, and 5 identified as core protein I, cytochrome b, cytochrome c_1, and the iron–sulfur protein, respectively, were highly labeled by [^{35}S]DABS; whereas, bands 6–9, containing the proteins of low molecular mass, were relatively lightly labeled. In contrast, bands 2, 6, and 7 were most highly labeled by [^{3}H]succinic anhydride with less, but still significant, labeling of bands 1, 3–5. It is also noted that the labeling by both reagents of contaminating proteins of high molecular mass was greater than expected on the basis of dye binding. The densitometer-scan of the stained gel yielded a significantly decreased staining of the band containing the iron–sulfur protein. This appears to be a result of an inadvertent loss of the iron–sulfur protein during processing of the subunits of the complex for electrophoresis as indicated by a concomitant lowered labeling of band 5.

It is difficult to determine the primary factor controlling the labeling accessibility of the Complex III subunits to succinic anhydride and DABS. Not only do the compounds differ in polar and ionic properties, but they differ in the type of reactive groups modified on the polypeptide. Therefore, both accessibility and reactivity factors must be considered. Cytochrome b was labeled by [^{35}S]DABS to a greater extent than by [^{3}H]succinic anhydride even though the opposite normally would be expected because of the relatively nonpolar properties of the cytochrome.

Labeling by [^{3}H]Succinic Anhydride of Subunits of Complex III Depleted of the Iron–Sulfur Protein

In this experiment, the differential labeling by [^{3}H]succinic anhydride was applied to determine the effects of removal of the iron–sulfur protein on
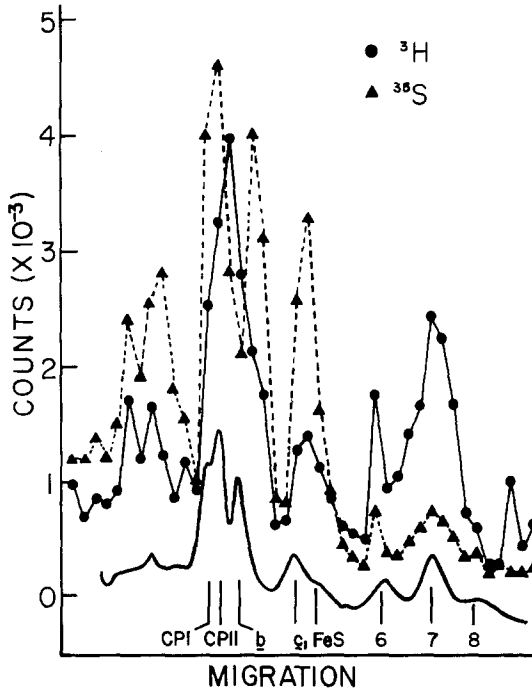
Fig. 1. Comparative labeling of the subunits of Complex III by [^{3}H]succinic anhydride and [^{35}S]DABS. Complex III (1.5 mg in 0.1 ml amine-free buffer) was stabilized by treatment with antimycin, then exposed to 8.7 nmol [^{3}H]succinic anhydride (2.4 mCi/nmol) added as a solution in 5 μl acetone. After 30 min the sample was exposed to [^{35}S]DABS (70 nmol, 100 μCi/nmol) as a solution in 5 μl water. After 30 min, unreacted DABS was decomposed by addition of a small pinch of Na dithionite. SDS-PAGE and measurements of radioactivity in individual gel slices were accomplished as described in Materials and Methods. ●, Radioactivity of 1 mm gel slices due to [^{3}H]succinic anhydride labeling; ▲, Radioactivity due to [^{35}S]DABS labeling. The continuous trace represents a spectrophotometric scan of the stained gel prior to slicing.

the accessibility to succinic anhydride of the remaining subunits of the complex. This experiment was considered feasible because the iron–sulfur protein can be selectively extracted from the antimycin-inhibited or the reduced complex by bile salt–ammonium sulfate mixtures or guanidinium salts with no apparent harm to the remaining quaternary structure of the complex. This is indicated by the capability of reconstitution of enzymic activity upon recombination of the depleted complex with the purified iron–sulfur protein (Trumpower and Edwards, 1979; Engel *et al.*, 1983). Scans of the stained PAGE gels prepared from intact, antimycin-treated Complex III and from antimycin-treated Complex III depleted of the iron–sulfur protein are shown in Fig. 2. Although the scan shows removal of only 50% of the iron–sulfur protein, the remaining iron–sulfur protein probably represents
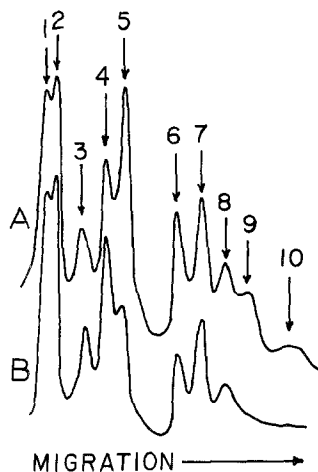
Fig. 2. Gel scans showing the depletion of the iron–sulfur protein after incubation of Complex III with a mixture of bile salt and ammonium sulfate. Complex III (1.35 mg in 0.05 ml amine-free buffer) was stabilized with 5 nmol antimycin (dissolved in 1 μl ethanol) and then incubated at 0°C with 5% Na taurocholate plus 25% saturation in ammonium sulfate. Scan A: Complex III precipitated by acetone immediately after addition of detergent–salt mixture. Scan B: Sample of Complex III incubated with the detergent–salt mixture for 3 hr after which precipitated protein was removed by sedimentation. Complex III in both samples, after precipitation in 90% acetone and recovery by sedimentation, was subjected to SDS-PAGE, staining, and scanning procedures as described previously (Ho *et al.*, 1985).

protein split from the complex but not precipitated under the conditions (pH 8.0) used, as judged from the rate of loss of antimycin sensitivity in the cleavage reaction (Rieske *et al.*, 1967). It is also noted that the components making up gel bands 9 and 10 were lost during the incubation of Complex III with taurocholate plus ammonium sulfate. We (unpublished) and others (Clejan *et al.*, 1984) have identified phospholipids as components with mobilities and staining similar to that of band 10 of this study, although proteolipids may also be implicated (Ho, 1979). However, in either case the low molecular mass as well as the solubility in polar organic solvents of the components making up band 10 easily explains their loss during the preparative procedures of SDS-PAGE.

Figure 3 shows the relative labeling of the polypeptide subunits of Complex III by exposure to [^{3}H]succinic anhydride after cleavage of the iron–sulfur protein from Complex III by sodium taurocholate plus ammonium sulfate (Fig. 3B) in comparison with the labeling of the subunits in a control sample of the intact complex (Fig. 3A). To insure identical conditions of exposure of the complex to succinic anhydride, the control sample of complex differed from the depleted sample only in length of incubation with the dissociating mixture, being essentially zero for the control sample. As
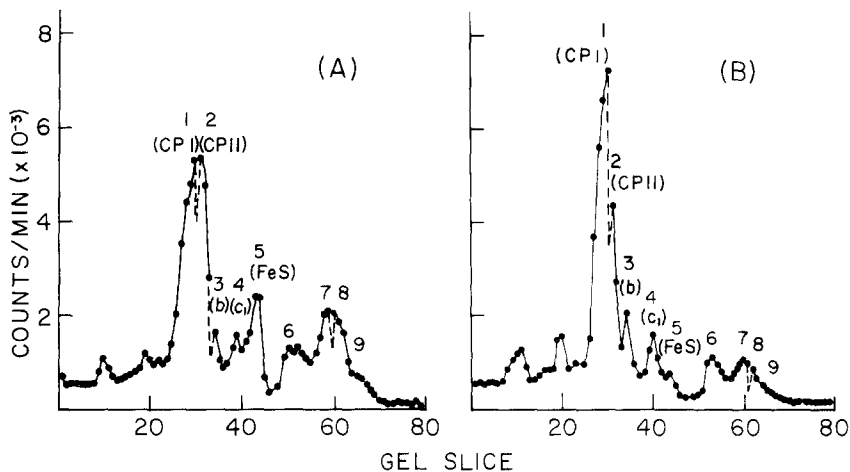
Fig. 3. Effects of selective removal of the Rieske iron–sulfur protein on the labeling by [^{3}H]succinic anhydride of the remaining subunits of Complex III. Complex III was treated as described for Fig. 2 at 0°C except that immediately after addition of ammonium sulfate the control sample (A) was treated with [^{3}H]succinic anhydride (8.7 nmol in 5 μl acetone, 2.4 mCi/ nmol) before being precipitated in 90% acetone; Sample (B) was treated identically except it was incubated for 3 hr after which precipitated iron–sulfur protein was removed by sedimentation prior to treatment with [^{3}H]succinic anhydride. After exposure to [^{3}H]succinic anhydride for 30 min the samples of Complex III were recovered and subjected to SDS-PAGE and measurements of radioactivity in individual gel slices as described in Materials and Methods. Dashed regions between bands 1 and 2 and bands 7 and 8 indicate regions of separation detected visually in the stained gels.

expected, the labeling of the PAGE band 5 containing the iron–sulfur protein was greatly reduced due to depletion of this subunit protein. However, band 1, containing core protein I, was significantly increased in labeling. This apparent increased accessibility of core protein I to succinic anhydride can be explained by a greater surface exposure upon removal of the neighboring iron–sulfur protein. This interpretation would be in agreement with results of crosslinking experiments in which core protein I and the iron–sulfur protein were linked together by the bifunctional reagents disuccinimidyl tartrate and N,N′-bis(3-succinimidyloxycarbonylpropyl) tartaramide (Smith and Capaldi, 1977; Smith et al., 1978). Also significantly decreased in labeling were the low-molecular-mass polypeptides associated with PAGE bands 7–9. The lowered labeling of band 9 can be attributed to extraction of band 9 polypeptide along with the iron–sulfur protein during incubation of the complex with taurocholate plus ammonium sulfate. However, since the polypeptides of bands 7 and 8 did not dissociate from the complex under these conditions, they must have become less accessible to attack by succinic anhydride upon removal of the iron–sulfur protein. In the absence of a better

explanation, this suggests that a significant conformational change associated with removal from the complex of the iron–sulfur protein and the polypeptide comprising band 9 may have occurred.

The labeling of band 4, containing cytochrome c_1, appeared relatively unaffected by removal of the iron–sulfur protein. However, the overlapping of bands 4 and 5 together with the low degree of labeling of band 4 could obscure an actual increase in labeling of band 4 subsequent to removal of the iron–sulfur protein (band 5). Assuming the presence of peptide groups reactive to succinic anhydride on the cytochrome c_1 interface with the iron–sulfur protein, an absence of any effect of removal of the iron–sulfur protein on the accessibility of cytochrome c_1 to succinic anhydride would be surprising in view of the apparent close relationship with respect to electron transfer between cytochrome c_1 and the iron–sulfur protein.

Use of Dual Labeling by ^{14}C and ^3H-Labeled Succinic Anhydride as a Probe of Redox-Mediated Conformational Transitions in the Subunits of Complex III

Because of the evidence linking conformational transitions of Complex III with changes in redox state (see Introduction) we have tested the effects of reduction on the patterns of subunit labeling by $[^{35}S]DABS$ and $[^{14}C]$succinic anhydride. Although Smith and Capaldi (1977) reported no change in labeling of Complex II subunits by $[^{35}S]DABS$ after reduction of the complex, our observations indicated that DABS, because of its strong oxidizing properties, was inappropriate as a reagent for labeling of the reduced complex. Therefore, we have conducted a similar experiment utilizing succinic anhydride as the labeling reagent.

A preliminary experiment had shown differences in labeling patterns of subunits in oxidized and reduced Complex III even when $[^3H]$succinic anhydride was added after the complex was dissociated in the sodium dodecyl sulfate medium (data not shown). This suggested that the presence of dithionite altered either the recovery of subunit polypeptides in the SDS-PAGE system or their reactivity to succinic anhydride even while in their presumably rodlike configuration when associated with the detergent (Reynolds and Tanford, 1970). Evidently, the second explanation applies because densitometer scans of SDS-PAGE gels of oxidized and reduced Complex III showed no significant differences in recovery of the different subunits (data not shown). To compensate for artifacts of this type, as well as to normalize variables such as molecular size and difficultly controlled variables in SDS-PAGE and gel slicing, a dual-labeling procedure was employed in which the oxidized or reduced complex was first exposed to $[^{14}C]$succinic anhydride while intact, then dissociated in the sodium dodecyl sulfate medium before

exposure to [^{3}H]succinic anhydride. The results of this experiment are shown in Figs. 4A–D.

The major difference, both qualitative and quantitative, between the subunit labeling patterns of oxidized and reduced Complex III are evident in core proteins I and II represented by gel bands 1 and 2. With the reduced complex, core protein I displayed a higher electrophoretic mobility relative to that of core protein II than with the oxidized complex. However, because this effect persisted with both the intact and dissociated complex labeled with ^{14}C and ^{3}H, respectively, and was not observed with unlabeled Complex III when reduced, it cannot be attributed to either conformational transitions or an effect of the reductant on the SDS-PAGE system. Significant differences in magnitude of labeling of bands 2, 4, 5, and 7 by [^{14}C]succinic anhydride were evident between oxidized and reduced Complex III (compare Figs. 4A and C). However, a significant change was noted only in band 2 when the ratios ^{14}C/^{3}H were considered (compare Figs. 4B and D). This difference between oxidized and reduced complex in the labeling of band 2 could not be explained by the greater overlapping of bands 1 and 2 in the reduced complex. This would indicate that only core protein II had undergone an apparent change in conformation detectable by the dual succinic anhydride probe.

In addition, the plots in Figs. 4B and D indicate that the magnitude of the labeling ratios ^{14}C/^{3}H can be a measure of the relative accessibilities or reactivities of the subunits of Complex III to succinic anhydride. On this basis, SDS-PAGE bands 2, 5, and 7 representing core protein II, the iron–sulfur protein, and an antimycin-binding protein (Ho et al., 1985), respectively, were the subunits most accessible to succinic anhydride. Cytochrome c_1, the subunit composing band 4, was next in order of accessibility. Of possible significance are apparent shoulders or peaks corresponding to bands 5 and 7 that may indicate the presence of two additional polypeptides of apparent molecular masses 23 and 12 kDa, respectively, that are not normally resolved electrophoretically. It must be emphasized that the apparent additional polypeptides associated with bands 5 and 6 could not be attributed to inhomogeneity of succinylation, which would yield polypeptides with variable numbers of negatively charged succinyl groups. Labeling of Complex III with less than stoichiometric amounts of succinic anhydride would make the possibility of polypeptides containing more than a single succinyl group unlikely. Also, the possibility that band 5 contains two polypeptides is supported by a resolution of two polypeptides from band 5 when subunits of Complex III were separated by SDS-PAGE in two dimensions employing 6 M urea in addition to sodium dodecyl sulfate in the second dimension (Ho and Rieske, 1983). In addition, the isolation of two cytochrome c_1-associated polypeptides have been reported, one of which, having an apparent molecular
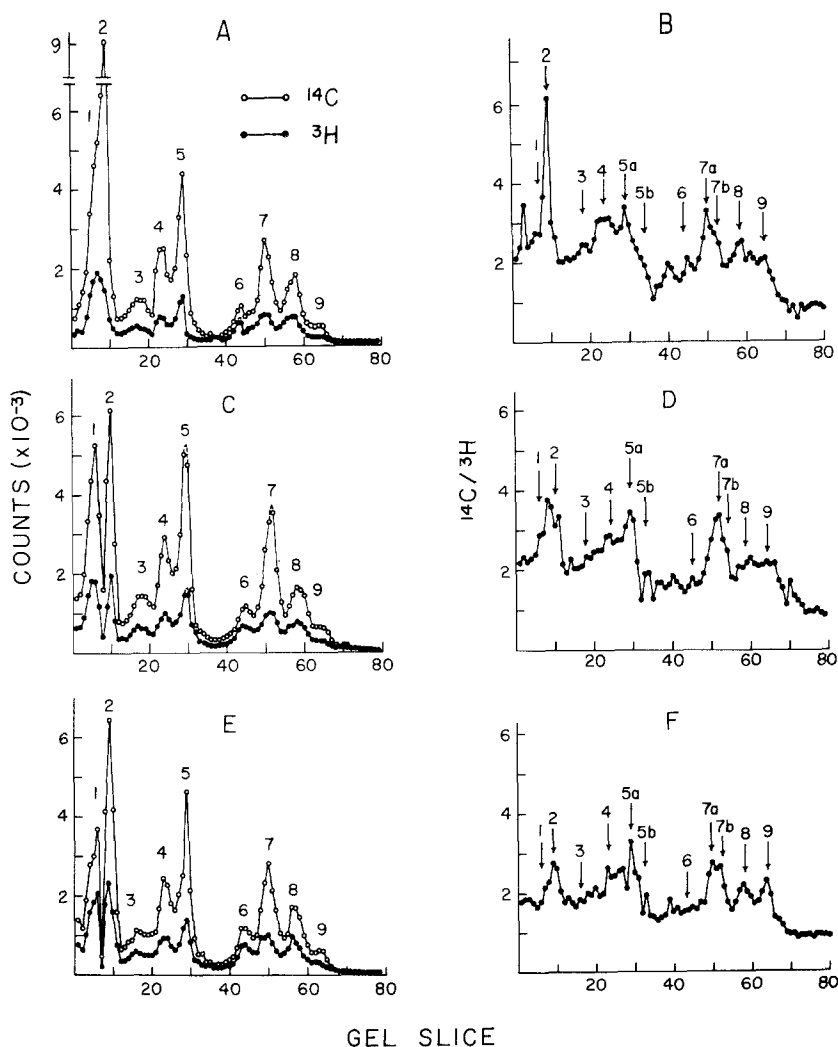
Fig. 4.   Effects of changes of oxidation state and antimycin on the dual labeling of subunits of intact and dissociated Complex III by [^{14}C]succinic anhydride and [^{3}H]succinic anhydride, respectively. Complex III (1.0 mg samples in 0.05 ml amine-free buffer) was treated with 5 μl of 50 mM K ferricyanide. Individual samples were treated as follows: A and B, reduced with dithionite; C and D, left oxidized; E and F, left oxidized and treated with 6 nmol antimycin in 6 μl alcohol. The samples were then exposed for 30 min (0°C) to [^{14}C]succinic anhydride (ca. 0.4 nmol, 8 μCi ^{14}C in 10 μl DMSO) after which the samples were precipitated and sedimented in 90% acetone. The pellets were rinsed with water and then dissolved and incubated 4 hr in dissociated buffer containing 10% SDS (see Materials and Methods). The solutions were treated with [^{3}H]succinic anhydride (ca. 8.7 nmol, < 250 μCi ^{3}H in 5 μl acetone) for 30 min after which any unreacted succinic anhydride was removed by incubation with 2 mg solid DTE. SDS-PAGE and measurements of radioactivity in individual gel slices were accomplished as described in Materials and Methods.

mass of 11 kDa based on SDS-PAGE mobility, could possibly be incorporated in band 7 (Wakabayashi *et al.*, 1982; Schägger *et al.*, 1983).

Effects of Antimycin on the Dual Labeling of Subunits of Complex III by ^{14}C and ^{3}H-Labeled Succinic Anhydride

Because antimycin exerts an effect similar to reduction in stabilizing
Complex III against cleavage by dissociating reagents such as bile salts plus
ammonium sulfate or guanidinium salts, we tested its effects on the accessibility of Complex III subunits to succinic anhydride. Utilizing the procedure of
the previous section, oxidized Complex III was compared with the antimycin-
treated complex. The results of this experiment are shown in Figs. 4C–F.
Significant changes in $^{14}C/^{3}H$ labeling ratios are evident for core protein I
(band 1) and band 7a, an antimycin-binding subunit (Ho *et al.*, 1985).
Smaller changes in the $^{14}C/^{3}H$ ratio are seen for core protein II (band 2), and
cytochromes b and c_1 (bands 3 and 4); however, the significance of these
smaller changes is questionable until statistics on a number of identical
determinations can be performed.

Discussion

A number of studies utilizing protein-modifying reagents as probes of
subunit arrangement in Complex III have been reported. These studies,
especially with purified Complex III, have yielded conflicting results with
respect to relative surface exposure of the subunit polypeptides to the various
probes as well as with separate studies utilizing the same probe. Possible
explanations of these discrepancies may involve variability of lipid and
detergent content of different preparations of Complex III, secondary subunit modifications of subunits perturbed in reactivity by the primary modification, and lack of normalization of subunit modifications to the relative
sizes of the subunits in addition to the experimental variables associated with
the SDS-PAGE and radioactivity measurements.

By utilization of probes at substoichiometric-labeling concentrations to
avoid secondary subunit modification, together with differential modification, dual isotopic labeling of the probe, and use of a probe with neutral
polarity, it was hoped that the variables listed above could be minimized.
An example of difficulties in interpretation of subunit labeled by different probes is the dual labeling of Complex III by [^{3}H]succinic anhydride
and [^{35}S]DABS. Although all other conditions remained constant, the relative accessibilities (or reactivities) of subunits to the two probes were strikingly
different. It is surprising that cytochrome b, by other criteria considered the

most hydrophobic of the subunits, was more highly labeled with the water-soluble DABS than with the much more hydrophobic succinic anhydride. This suggests that the relative labeling of cytochrome *b* by the two probes was determined principally by the number of peptide groups reactive with each probe rather than by the accessibility of the cytochrome to the probes. The amino acid content of cytochrome *b* indicates a low content of lysine and cysteine residues that would specifically react with succinic anhydride, in comparison with the content of residues such as tyrosine, tryptophan, and histidine that would be most reactive with DABS (Anderson *et al.*, 1982).

The effects of removal of the iron–sulfur protein on the accessibility to [^{3}H]succinic anhydride of the remaining subunits present some difficulties in interpretation. The increased labeling of core protein I as mentioned previously can be explained by a removal of shielding by the adjacent iron–sulfur subunit. However, the observed decrease in labeling of the poly-peptides associated with PAGE bands 7 and 8 would suggest a change in conformation of the complex upon removal of the iron–sulfur protein. This conformational change would have to be readily reversible to account for the ease in which the activity of the depleted complex can be reconstituted with purified iron–sulfur protein (Trumpower *et al.*, 1979; Engel *et al.*, 1983). Alternatively, it is possible that the decreased accessibility of subunits 7 and 8 was caused by removal of the polypeptide associated with PAGE band 9, which was removed from the complex along with the iron–sulfur protein. However, it must be pointed out that this experiment was not performed with the dual-labeling controls as utilized in subsequent experiments. Therefore, the significance of nominal changes in subunit labeling observed after removal of the iron–sulfur protein is yet uncertain.

The results from experiments utilizing dual labeling of oxidized, reduced, and antimycin-treated Complex III with ^{14}C- and ^{3}H-labeled succinic anhydride demonstrate the increased sensitivity of subunit labeling resulting from normalization of such factors as subunit size and variables associated with the SDS-PAGE and gel-slicing procedures. This increase in sensitivity is demonstrated by the possible detection of secondary PAGE bands 5b and 7b that are usually not resolved by staining procedures. Whether these bands represent distinct subunits of Complex III, chemically modified variants of subunits 5 and 7, or contaminating proteins is yet uncertain. Band 7b may represent the protein associated with cytochrome c_1 that is reported to function in the binding of cytochrome *c* to cytochrome c_1 (Wakabayashi *et al.*, 1982).

The results of reduction of Complex III on subunit labeling by ^{14}C and ^{3}H-labeled succinic anhydride are difficult to correlate with other studies. With the exception of core protein II the subunits exhibited at most minor changes in labeling upon reduction of the complex. However, under similar conditions the rate of digestion .of the iron–sulfur protein by trypsin was

reported to be significantly increased in the reduced complex (Ball *et al.*, 1977; Baum *et al.*, 1967). In contrast, with yeast Complex III it was reported that reduction decreased the digestion rate of the iron–sulfur protein and a polypeptide of $M_r = 17,000$ designated as subunit VI (Sidhu and Beattie, 1982). It was also reported that chymotrypsin rapidly digested cytochrome b, cytochrome c_1, iron–sulfur protein, and subunit VII, either in the oxidized or reduced state of the complex. The core proteins and subunits VI were uniformly resistant to digestion by chymotrypsin.

Reduction of the complex has been found to stabilize the complex against cleavage by bile salts plus ammonium sulfate or guanidinium salts (Rieske *et al.*, 1967). This effect was attributed to a redox-linked conformational change that shields the quaternary structure of the complex from the dissociating reagent (Rieske, 1976). If this mechanism is correct, it could be expected that reduction of the complex would significantly decrease the accessibility of certain key subunits to succinic anhydride. Because this effect was not observed in this study, it is suggested that reduction protects Complex III against dissociation not by lowering the accessibility of the subunits, but by changing the conformation of the quaternary structure to a form more resistant to dissociation, even though the subunits remain accessible to the dissociating reagents. This concept is consistent with the observed effect of reduction on the cleavage of Complex III by taurocholate plus ammonium sulfate (Rieske *et al.*, 1967). In this case it was found that the actual cleavage of the complex followed an initial rate-limiting process that appeared to involve the penetration of the taurocholate into the quaternary structure. Reduction of the complex inhibited the cleavage reaction but not the initial assumed penetration process.

The binding of antimycin to Complex III appeared to exert a more generalized effect on the succinic anhydride-labeling pattern than reduction. The observed decrease in labeling of core protein I, PAGE band 7a, and possibly cytochromes b and c_1 indicate either an antimycin-induced tightening of quaternary structure or a direct shielding by the bound antimycin. Because a polypeptide comprising band 7 has been implicated in the binding of antimycin (Ho *et al.*, 1985), a direct shielding of this polypeptide by bound antimycin may explain the decrease in labeling.

In similarity to reducing reagents, antimycin stabilizes Complex III against cleavage by certain dissociating reagents. However, the labeling studies reported here suggest that the stabilization by reducing agents and antimycin involve different mechanisms. This concept is supported by the early observation that reduction, but not antimycin, protected Complex III from guanidine-induced cleavage when the complex was stored in a frozen medium (Rieske *et al.*, 1967).

In general, the results of this study have demonstrated the potential usefulness of differential and dual labeling in the study of subunit architecture and conformational transitions in multisubunit complexes such as Complex III. Also, the variability of subunit modification observed with different probes furnishes these techniques with wide versatility. The study reported here suffered from the time-consuming process of the measurement of labeling profiles by the counting of individual gel slices. The recent development of autoradiofluorography and sensitive densitometry should greatly improve the usefulness of differential labeling. However, application of these newer techniques to dual-labeling experiments will require further development.

Acknowledgments

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