Characterization of the Covalent Cross-links of the Active Sites of Amidinated Cytochrome \( b_5 \) and NADH:Cytochrome \( b_5 \) Reductase*

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Preparations of amidinated cytochrome \( b_5 \) and cytochrome \( b_5 \) reductase, cross-linked by using a soluble carbodiimide to promote the formation of covalent bonds between carboxyl groups of the hemeprotein and nucleophilic residues of the flavoprotein at the surfaces involved in protein-protein contacts during electron transfer, have been used to characterize the charge pair interactions that occur during electron transfer between the free proteins. Sequence analyses of tryptic, V8 protease-, and Asp-N protease-generated peptides show that the heme propionyl carbonyl group at the surface of the cytochrome forms an ester bond with Ser\(^{192} \) of the reductase, thus implicating Lys\(^{128} \) as the normal participant in ionic bonding between the active sites of the two proteins. Moreover, Lys\(^{41} \) and Lys\(^{125} \) directly form amide bonds with carboxyl residues on the active-site surface of the cytochrome. In the case of Lys\(^{41} \), this involves Glu\(^{62} \) and/or Glu\(^{66} \), and Glu\(^{17} \) and/or Glu\(^{28} \) for Lys\(^{125} \), again implicating these residues as the groups that form charge pairs during normal interactions between the active sites of the two proteins.

Cytochrome \( b_5 \) plays a central role in the transfer of electrons from two flavoproteins (1–3) to several specific terminal electron acceptors on the cytoplasmic surface of the liver endoplasmic reticulum (4–8). Both this hemeprotein and the NADH-specific flavin-protein cytochrome \( b_5 \) reductase are amphiphatic molecules composed of globular, hydrophilic, catalytic domains linked through short flexible sequences to the membrane-anchoring hydrophobic domains that serve to orient the catalytic sites at the membrane-aqueous interface to permit rapid electron transfer sequences (1, 9). A recent update of the amino acid sequences of cytochrome \( b_5 \) from several species has appeared (10). There is also considerable evidence (11–13) that cytochrome \( b_5 \) as well as other electron transport components of these oxidative pathways in the endoplasmic reticulum undergo relatively free lateral movement to facilitate interactions between the hemeprotein and a spectrum of electron donors or acceptor proteins.

The x-ray crystallographic studies of Mathews and Czerwinski (14) revealed that a striking feature of the structure of the heme peptide (the tryptic generated soluble catalytic fragment of cytochrome \( b_5 \)) segment is the crescent-shaped array of carboxyl groups around the exposed propionyl carboxyl of the heme edge that is oriented on the surface of the protein. Salemme (15) used a best-fit computer program of this data and similar crystallographic data for cytochrome \( c \) to suggest that electron transfer between the two proteins involves charge pairing between carboxyl residues Glu\(^{25} \), Glu\(^{18} \), and Asp\(^{44} \) and the exposed heme propionate of cytochrome \( b_5 \) with complementary residues surrounding the cytochrome \( c \) heme edge. Subsequent studies by Daily and Strittmatter (16, 17) utilized selective carboxylate group modification to implicate carboxyl groups in this crescent-shaped, active-site region of amidinated cytochrome \( b_5 \) in interactions with NADH:cytochrome \( b_5 \) reductase (16), NADPH:cytochrome \( P-450 \) reductase (17), and stearyl-CoA desaturase (17). A direct examination of such a reactive complex between amidinated cytochrome \( b_5 \) and NADH:cytochrome \( b_5 \) reductase inserted in liposomes utilized covalent cross-linking between carbodiimide-activated carboxyl groups of the amidinated cytochrome to presumed complementary \( \varepsilon \)-amino groups of lysyl residues in the reductase (18). The isolated cross-linked amidinated cytochrome \( b_5 \) reductase preparations showed all of the kinetic properties, i.e. rapid electron transfer from NADH to reductase FAD to cytochrome \( b_5 \), consistent with cross-linking at the surfaces involved in protein-protein contacts in an orientation similar to that assumed during electron transfer between the free proteins. In all of these studies (16–18), amidinated preparations of cytochrome \( b_5 \) were employed to eliminate the lysyl \( \varepsilon \)-amino groups as possible nucleophiles that might react subsequently with EDC-activated carboxyl groups. This modification retains the positive charge on these residues and has no effect on the catalytic reaction of the free cytochrome or cytochrome cross-linked to the reductase as indicated in these references.

We have now characterized the cross-links formed between the amidinated hemeprotein and flavoprotein (the tryptic generated soluble catalytic fragment of NADH:cytochrome \( b_5 \) reductase) by isolating three cross-linked peptide pairs each involving one reductase peptide sequence and one hemeprotein sequence. These data suggest that interactions between amidinated cytochrome \( b_5 \) and cytochrome \( b_5 \) reductase involve charge pairing between 3 lysyl residues of the flavoprotein with the array of carboxylate groups surrounding the heme edge of the hemeprotein.

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\* The abbreviations used are: EDC, 1-ethyl-3-(2-dimethylamino-propyl)carbodiimide hydrochloride; HPLC, high pressure liquid chromatography; DMPC, dimyristoylphosphatidylcholine; SDS, sodium dodecyl sulfate.
Amidinated Cytochrome b$_5$/Cytochrome b$_5$ Reductase Cross-links

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

The initial tryptic digestion of AHPXFP (the cross-linked preparation of amidinated heme peptide and flavoprotein) (Figs. S3-S5 and Tables S1-SIV) served to identify three cross-linking sites in the catalytic flavopeptide segment of cytochrome b$_5$ reductase with active-site carboxylate residues of amidinated cytochrome b$_5$, identified by earlier studies (16). Because the AHP segment in peptide T-2 begins with either Tyr$^{1}$ or Thr$^{12}$ and the FP portion ends with Tyr$^{129}$ (all sensitive chymotryptic sites), this digestion with a preparation of trypsin was probably contaminated by a low level of active chymotrypsin. Nevertheless, sequence analysis (Table SII) clearly identifies the cross-linked residue of peptide T-2 as Lys$^{59}$ of the reductase (19). This presumably forms an amide bond during the cross-linking reaction with either Glu$^{67}$ and/or Glu$^{88}$, identified as active-site residues (16), which are part of peptide T-2 but well beyond the limited sequence analysis.

Peptide T-4 has both the amino acid composition (Table SII) and the sequence expected for cross-linked segments Ser$^{26}$ to Arg$^{59}$ of the reductase and Glu$^{59}$ to Arg$^{70}$ of the cytochrome (Table SIII). In this case, the absence of Lys$^{19}$ (19) at cycle 13 identifies the reductase residue as the participant in the cross-link. The complementary carboxylate, however, is not defined clearly as a single residue. Since the AHP residues at cycles 1, 9, and 13 have been identified as active-site residues Glu$^{59}$, Glu$^{67}$, and Asp$^{64}$ of cytochrome b$_5$ (16), respectively, and are clustered near one another, Lys$^{59}$ may have formed a population of amide bonds involving any of these carboxyl groups in individual AHPXFP molecules. This would at least in part reflect the relative activation of these residues during the brief reaction with carbodiimide in the cross-linking procedure. In addition, the Edman reaction involving either N-terminal Glu or Asp residues involved in cross-links to lysyl ε-amino groups may undergo cleavage during a phase of the Edman degradation.

Peptide T-5 represents the cross-link between reductase and the heme propionyl carboxyl group identified earlier as an active-site residue (16). Fig. S2 shows the 400 nm absorption of the fraction used to obtain peptide T-5 in Fig. S5. The high 280 nm absorbance of peptide T-5 in the HPLC record expected for heme was also seen. The sequence analysis in Table SIV identifies Ser$^{58}$ of the reductase (19) as the cross-link to the heme propionyl group. This presumably reflects both steric factors and the deamidability of Ser$^{58}$ in reacting with the carbodiimide-activated carboxyl group and suggests that normal interaction between the cytochrome and flavoprotein, involving charge complementation, included heme carboxyl ionic pairing with the neighboring ε-amino group of Lys$^{59}$.

To further characterize the carboxylate groups of the cytochrome involved in cross-linking to reductase at both Lys$^{59}$ and Lys$^{129}$, tryptic digestion with a preparation proven completely devoid of chymotryptic activity was used to isolate the two cross-linked peptides T-6 and T-7 (Figs. S6 and S7) with amino acid compositions expected for the tryptic peptides (Table SV). Peptide T-6, the expected cross-link between residues 151 of the cytochrome and residues 120-142 of the reductase, was then first amidinated to block subsequent Edman degradation beginning with Phe$^{129}$ of the reductase segment. This was followed by V8 protease digestion to produce peptide V5-1 (Fig. S8) with the amino acid composition expected for reductase Phe$^{120}$ to Glu$^{121}$ (19) cross-linked to amidinated cytochrome Glu$^{59}$ to Arg$^{59}$. Table SVI shows that, with the N-terminal Phe blocked by amidination, only the segment derived from the cytochrome undergoes Edman degradation. This sequence includes Glu$^{67}$ and Glu$^{88}$ (cycles 6 and 7). Both of these residues appear to be involved in cross-linking to Lys$^{59}$ of the reductase. The yield of Glu at cycle 6 is significantly lower than that at cycle 7, indicating a significant carry-over in Glu release from residual Glu of cycle 6 at cycle 7. Moreover, the recoveries are significantly lower than those in Glu release at cycle 1, and there is a sudden decrease in yield at cycle 8. These are all consistent with the assumption that either Glu$^{67}$ or Glu$^{88}$ will cross-link with Lys$^{59}$ depending in part on the pattern of carboxyl activation by carbodiimide. In addition, the possible instability of the amide cross-link during Edman degradation cycles involving the N termini of these residues may significantly obscure the extent of cross-linking.

Peptide T-7 (Table SV) has the amino acid composition and sequence (Table SVII) expected for the cross-link between residues 52-72 of the cytochrome and residues 29-45 of the reductase. The absence of Lys at cycle 13 for the reductase sequence confirms the conclusion that the ε-amino group of Lys$^{59}$ participates in the amide cross-link. However, again, the carbonyl partner of the cytochrome segment is not clearly defined. This segment contains Glu$^{67}$, Glu$^{88}$, and Asp$^{64}$ at cycles 1, 9, and 13, respectively, and involved in amidinated cytochrome b$_5$ interactions with the reductase (16). Only cycle 1 shows a low yield of Glu, which was expected if a cross-linked residue remained intact during the Edman sequence analysis. To follow this characterisation further, a sample of peptide T-7 was subjected to Asp-N protease digestion to produce peptides cleaved on the amino side of labile Asp peptide bonds. Peptides A-1 and A-2, obtained by HPLC (Fig. S9), with the amino acid compositions shown in Table SVIII were subjected to sequence analysis as shown in Tables SIX and SX. Peptide A-1 is a mixture of the reductase segment Asn$^{37}$ to Arg$^{59}$ with the missing Lys$^{59}$ identified as the cross-linking site again as a result of Asp-N protease cleavage between Glu$^{66}$ and Asn$^{37}$. The two cytochrome sequences Asp$^{57}$ to Glu$^{66}$ and Glu$^{66}$ to Glu$^{88}$ are two expected Asn-N protease peptides with incomplete cleavage at Asp$^{64}$. The amount of reductase segment suggests that both cytochrome segments are cross-linked to Lys$^{59}$ of the reductase. For the shorter Asp$^{64}$ to Glu$^{88}$ sequence, Glu$^{66}$ at cycle 4 has a lower yield, consistent with a cross-linked residue, whereas Asp$^{64}$ is excluded since it was an Asp-N protease cleavage site. In the case of the longer Glu$^{66}$ to Glu$^{88}$ segment, only Glu$^{66}$ at cycle 1 appears to exhibit a lower yield as the first residue of the sequence. Further and clearer evidence for Glu$^{66}$ cross-linking to reductase Lys$^{59}$ is seen in the sequence data for peptide A-2 (Table SX). In this case, Glu$^{66}$ expected at cycle 1 of the sequence Glu$^{52}$ to Glu$^{66}$ is completely missing, thus clearly identifying this residue as one carboxyl donor in amide cross-linking with Lys$^{59}$ (cycle 3) of the reductase segment. The 9-residue segment of amidinated cytochrome b$_5$ (Asp$^{64}$ to Arg$^{70}$) is a minor contaminant that probably is not involved in cross-linking. It is only 2 residues shorter than the 11-residue cross-linked peptide, and the Asp at cycle 1 cannot be a cross-link since it was Asp-N protease-sensitive and, at cycle 8, Asp$^{60}$ has previously been shown to be relatively unreactive to carbodiimide (16). Furthermore, the crystallographic data place the carboxylate...
FIG. 1. Graphics for active-site carboxyl residues of cytochrome \(b_6\) and cross-linked and/or presumed charge-paired residues of cytochrome \(b_5\) reductase. The graphics for cytochrome \(b_6\) were ChemX-designed and distributed by Chemical Design Ltd. and generated by David W. Chester and Mark Trumbore (University of Connecticut Health Center). Lys$^+$ and Lys$^-$ of the reductase were cross-linked by the \(\epsilon\)-amino groups and are presumably normally involved in charge pairing with neighboring carboxylates of cytochrome \(b_6\). Ser$^{166}$ of the reductase was cross-linked to the heme propionyl carboxyl group, indicating that Lys$^{166}$ is normally charge-paired during interaction with free cytochrome. The sequence data for calf cytochrome \(b_6\) are given in Ref. 10, and the primary structures of the reductase in Ref. 19.

group of this residue outside of the defined crescent of carboxyl groups surrounding the heme crevice (14, 16).

In several respects, previous work (16-18) and the experimental design used here aid in the interpretation of these data. It is clear from EDC activation and reaction with small nucleophiles (16, 17) that several carboxyl groups surrounding the heme crevice of the cytochrome are most reactive under the conditions that we have employed for EDC activation and that reaction of these groups with nucleophiles drastically inhibits interaction with the reductase. Combined with the initial cross-linking data (18) and the study of Salamme (15) with cytochrome \(b_6\) and cytochrome \(c\), these results support the conclusion that EDC activation is directed to the active-site carboxyl groups of the cytochrome which form charge pairs with specific reductase lysyl residues in rapid electron transfer between the two proteins. However, because the EDC reaction at pH 7.0 is brief and the subsequent incubation at pH 9.5 to increase the concentration of uncharged \(\epsilon\)-amino groups as nucleophiles for covalent coupling decreases the stability of EDC-activated carboxyl groups, the yield of cross-link is relatively low. This analytical problem is exacerbated by the clustering of carboxyl groups around the heme crevice leading to multiple coupling possibilities and a spectrum of cross-links to further decrease the concentration of any one cross-link for analysis.

With these limitations in mind, we have employed Fig. 1 to summarize the implications of these data. The original crystallographic data on cytochrome \(b_6\) of Mathews and Czerwinski (14) were used to generate the graphics for the hemoprotein carboxyl groups surrounding the heme crevice of this protein as identified in previous studies (16, 17). The results presented here place the \(\epsilon\)-amino groups of 3 lysyl residues of cytochrome \(b_5\) reductase (lysines 41, 125 and 163) near the anionic residues of the cytochrome, as indicated in Fig. 1.

Carbodiimide activation of the heme propionyl carboxylate that appears on the surface of the cytochrome (14) results in cross-linking to Ser$^{166}$ of the flavoprotein. Although stereo- and/or the strength of nucleophilicity results in the formation of this ester bond, the \(\epsilon\)-amino group of the adjacent Lys$^{60}$ is the probable participant in charge pairing with this carboxyl group during normal catalytically productive interactions between the two proteins. Both Lys$^{41}$ and Lys$^{125}$ were clearly revealed as participants in similar charge complementation by the cross-linking data. However, in these two instances, two carboxyl groups appear to be sufficiently near either lysyl residue (Glu$^{62}$ and Glu$^{60}$ in the case of Lys$^{41}$ and Glu$^{125}$) to form amide bonds when either of the two glutamyl \(\gamma\)-carboxyl groups is activated during cross-linking of the two proteins.

Since we have previously shown (18) that the cross-linked amidated cytochrome and flavoprotein carry out rapid, normal electron transfer between the flavin and heme, these lysyl residues presumably can also form transient ion pairs with either of the identified glutamyl residues during electron transfer between the free, noncross-linked enzymes. This constitutes restrictive data that begin to define not only the reactive areas on the surface of the reductase, but also the precise structure that is required for the rapid transfer of electrons between the flavin and heme of the two proteins. With the earlier identification of Cys$^{260}$ (20) and Lys$^{161}$ (21) as residues involved in NADH interaction with cytochrome \(b_6\) reductase, there are now 5 known residues in the primary structure of the flavoprotein which participate in flavin reduction by the electron donor, (NADH) and reoxidation by the electron acceptor (cytochrome \(b_6\)). Construction of a bacterial expression vector for this flavoprotein from its known complete primary structure (19) should permit the application of specific site-directed mutagenesis experiments to evaluate the contribution of each of these presumed active-site residues to the binding and/or mechanism of the electron transfer sequence. Moreover, either completion of the initial x-ray crystallographic analysis of the catalytic domain of cytochrome \(b_6\) reductase (22) or the generation of x-ray crystallographic data on AHFXPF should provide the most definitive understanding of the orientation of NADH, FAD, heme, and protein structures during this complex oxidative reaction involving the transition from two to one electron acceptors.

REFERENCES

Continued on next page.
**Amidinated Cytochrome b5/Cytochrome bs Reductase Cross-links**

**Materials and Methods**

Cytochrome b5 and cytochrome bs reductase were purified from Black Angus steer liver as described (18,22). NASID-cytochrome b5 reductase and NASID-Cytochrome bs reductase activities (24) were used to monitor amidination concentrations. Cytochrome bs concentrations were determined by the absorbance at 460 nm (9) or by using fluorimetric reading (25) to determine the reactivity present when radiolabeled cytochrome was used. Synthetic DMPC vesicles were prepared by sonication in 10 mM Tris-acetate buffer, pH 7.0, and 0.1% sodium azide. Following the addition of solid LDC to the 105,000 × g pellet obtained from the previous centrifugation step, the reaction mixture was incubated at 90°C for 30 min to destroy the reductase. The reaction mixture was subsequently analyzed on an agar gel electrophoresis apparatus as described by Lammi (26). Ethidium bromide accumulation was prepared as described previously (18). Ammonium acetate was prepared as described previously (18). All enzymes were stored at -20°C. DMPC, Sephadex G-25, Sephadex G-25-150, and G-15-120, sodium azide, and 2-mercaptoethanol were purchased from Pharmacia, and Tris was purchased from Westerham, and V-8 protease and Asp-N protease from Boehringer Mannheim.

**Results**

High Performance Liquid Chromatography was performed on a Waters HPLC system consisting of a 5 µm, 25 cm × 0.46 cm, aluminocarbomethoxylate detector, a 481 variable wavelength detector and a chart recorder, with an injection valve broad peak and the flow rate of V-of 150 µm (500 µm) and a Waters C18 prepouch (3.0×3.9 mm) column was used for the separation of proteins by high-performance liquid chromatography. Amidinated cytochrome, ubiquitously at a specific activity of 12500.1000 15N-labeled leucyl residues was stored at 20°C.

**Amidination of cytochrome bs**

Cytochrome bs5 was amidinated using the procedure described previously (18), usually with [15N] labeled reagent. This procedure completely amidates lysyl residues as indicated by the absence of reactions with trichloroacetic acid. Amidinated cytochrome was, ubiquitously at a specific activity of 1230.000 leucyl residues was stored at -20°C.

**Cross-linking of amidinated cytochrome bs and cytochrome bs reductase**

A modification of the previous preparation of the cross-linked cytochrome and lysyl residues was employed to increase the yield of product. The initial yield of 20 mM DMPC vesicle preparation, at 30°C, containing amidated cytochrome-bs (100 µM) and reductase (35 µM) and 10 mM NASID in 10 mM Tris-acetate buffer, pH 7.0, and 0.025% sodium azide. Following the addition of solid LDC to the 105,000 × g pellet obtained from previous centrifugation step, the reaction mixture was immediately passed over a 3 × 45 cm column of Sephadex G-25-150 equilibrated with 10 mM sodium pyrophosphate buffer at pH 9.5 and 0.25% sodium azide, approximately 30 ml of the reaction mixture was collected at 1 ml/min to 15% DMSO and subsequently analyzed on an agar gel electrophoresis apparatus as described by Lammi (26). Ethidium bromide accumulation was prepared as described previously (18). Ammonium acetate was prepared as described previously (18). All enzymes were stored at -20°C. DMPC, Sephadex G-25, Sephadex G-25-150, and G-15-120, sodium azide, and 2-mercaptoethanol were purchased from Pharmacia, and Tris was purchased from Westerham, and V-8 protease and Asp-N protease from Boehringer Mannheim.

**Protection and Purification of, Soluble, AMIDOP**

An initial partial purification of cross-linked amidinated cytochrome bs/Cytochrome bs reductase involved the addition of 20% triton X-100 solution to the 50 µl of eluate at 4°C in a final concentration of 2%. All subsequent steps were at 4°C. Eight mg of potassium ferricyanide was then added to oxidize any reduced reductase or NASID that emerged in the void volume of the gel filtration step. This solution was passed over a 10 µm column packed with Sephadex G-25-150 equilibrated in 0.5% Triton X-100 and 10 mM Tris-acetate buffer, pH 8.1, to remove NASID from the void containing the proteins. Equal total volumes of eluate were then added to 10 µl of NAD-glycoenase equilibrated with 2% triton, 50 mM Tris acetate, 50 mM phosphate buffer, pH 8.1, and sufficient solid sodium hydroxide to maintain anaerobic conditions. Subsequent washing with approximately 20 ml of the same buffer removed all free amidinated cytochrome bs, from the column. Triton X-100 was then added to the column containing cytochrome bs isoenzymes column with approximately 40 ml of 0.3% sodium deoxycholate in 30 mM Tris-acetate, pH 8.1. The cross-linked cytochrome bs reductase, as free reductase were then eluted 100-200 mls of 0.3% sodium deoxycholate, 20 mM Tris-acetate buffer, pH 8.1, containing 10 mM potassium ferricyanide. NASID and potassium ferricyanide were removed from the protein solution by repeated concentration in centricon 30 tubes and addition of 0.3% sodium deoxycholate, 20 mM Tris-acetate buffer, pH 8.1. The final concentration of 2.5 ng/ml was prepared approximately 170-200 ml of amidinated cytochrome bs and 300-500 ml of reductase, the excess protoporphyrin over the protein representing free reductase.

To generate cross-linked amidinated horse heart protoporphyrin, 40 ml of solid NASID was added to 2.5 ml of nitrated protoporphyrin solution and 200 µM tripton was then added under nitrogen to a final molar ratio of 2:20 trytoproph, reduction, and incubated for 45-45 hours at 4°C.

**Purification of Cross-linked, AMIDOP**

The tryptic digestion of cross-linked protoporphyrin was terminated by addition of 30 µl of 100 mM phenethylmerinthylacetate fluoride in 10 mM Tris-acetate, at 0-4°C and the following isolation procedure was carried out at this temperature. The preparation was passed over a 40 ml Sephadex G-25-150 column equilibrated with 100 µM sodium acetate buffer, pH 8.1, to exclude protein fraction (approximately 6 ml) was loaded onto 3 ml of DEAE-cellulose (Whatman DE52) equilibrated with the same buffer. Washing the elution of 25 ml of of 0.3% sodium deoxycholate in 30 mM Tris-acetate, pH 8.1, 0.3% sodium deoxycholate, 75% NIC and 10 mM Tris-acetate buffer, pH 8.1, served to elute the sample peptide segments of both the cytochrome and ferrocyanide as free ferrocyanide. After removing all triton X-100 column with 20 ml of 0.2% sodium deoxycholate, 10 mM Tris-acetate buffer, pH 8.1, the column was used to remove most of the sodium deoxycholate from the column. Elution of the HPLC-purified in a concentrated band was used to elute the sodium acetate buffer, pH 8.1, to the sodium deoxycholate concentration of 20 ml with 20 mM Tris-acetate buffer, pH 8.1, in the concentration of 20 mls was then used to obtain approximately 50 mg of eluted cross-linked AMIDOP free of NaCl. The cross-linked AMIDOP was dialyzed in a low concentration of 1.0% sodium deoxycholate. The yield of the cross-linked AMIDOP was approximately 10% of the total AMIDOP protein as determined by the SDS-PAGE analysis. The yield of the cross-linked AMIDOP was approximately 10% of the total AMIDOP protein as determined by the SDS-PAGE analysis.
### Table 11A: Summary Analysis of All Data Table 11B

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* Indicates absence of data.

![Graphs and diagrams related to Table 11A and Table 11B.](http://www.jbc.org/)

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**Amidinated Cytochrome b₅/Cytochrome b₅ Reductase Cross-links**

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