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Chemical Cross-Linking Study of Complex Formation between Methylamine Dehydrogenase and Amicyanin from *Paracoccus denitrificans*†

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ABSTRACT: Two soluble periplasmic redox proteins from *Paracoccus denitrificans*, the quinoprotein methylamine dehydrogenase and the copper protein amicyanin, form a weakly associated complex that is critical to their physiological function in electron transport [Gray, K. A., Davidson, V. L., & Knaff, D. B. (1988) *J. Biol. Chem. 263*, 13987–13990]. The specific interactions between methylamine dehydrogenase and amicyanin have been studied by using the water-soluble cross-linking agent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Treatment of methylamine dehydrogenase alone with EDC caused no intermolecular cross-linking but did cause intramolecular cross-linking of this $\alpha_2\beta_2$ oligomeric enzyme. The primary product that was formed contained one large and one small subunit. Methylamine dehydrogenase and amicyanin were covalently cross-linked in the presence of EDC to form at least two distinct species, which were identified by nondenaturing polyacrylamide gel electrophoresis (PAGE). The formation of these cross-linked species was dependent on ionic strength, and the ionic strength dependence was much greater at pH 6.5 than at pH 7.5. The effects of pH and ionic strength were different for the different cross-linked products. SDS–PAGE and Western blot analysis of these cross-linked species indicated that the primary site of interaction for amicyanin was the large subunit of methylamine dehydrogenase and that this association could be stabilized by hydrophobic interactions. In light of these results a scheme is proposed for the interaction of amicyanin with methylamine dehydrogenase that is consistent with previous data on the physical, kinetic, and redox properties of this complex.

Paracoccus denitrificans, when grown on methylamine as the sole source of carbon and energy, synthesizes an inducible methylamine dehydrogenase which functions in the periplasmic space of this Gram-negative bacterium (Husain & Davidson, 1987). It catalyzes the oxidation of methylamine to formaldehyde and ammonia and donates electrons to an inducible periplasmic type I copper protein, amicyanin (Husain & Davidson, 1985). Methylamine dehydrogenase is an oligomeric protein with an $\alpha_2\beta_2$ structure and subunit molecular weights of 46 700 and 15 500 (Husain & Davidson, 1987). Each small subunit contains covalently bound pyrroloquinoline quinone (PQQ).[1] Amicyanin is a monomeric protein with a molecular weight of 15 000 (Husain & Davidson, 1985).

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PAGE, polyacrylamide gel electrophoresis; PQQ, pyrroloquinoline quinone.

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Growth of *P. denitrificans* on methylamine also induces the synthesis of a soluble periplasmic cytochrome *c*-551i (Husain & Davidson, 1986), which is another intermediate in the transfer of electrons from methylamine to the membrane-bound respiratory chain (Davidson & Kumar, 1989). Earlier studies from this laboratory have extensively characterized these redox proteins (Chen et al., 1988; Davidson, 1989; Davidson & Neher, 1987; Gray et al., 1986, 1988; Husain & Davidson, 1985, 1986, 1987; Husain et al., 1986, 1987; Lim et al., 1986; Sharma et al., 1988) and have provided oxidation–reduction midpoint potential (E_m) values of +100 mV for the PQQ in methylamine dehydrogenase (Husain et al., 1987), +294 mV for the copper center in amicyanin (Gray et al., 1986), and +190 mV for cytochrome *c*-551i (Gray et al., 1986). Kinetic studies established that amicyanin served as an obligatory intermediate in the transfer of electrons from methylamine dehydrogenase to cytochrome *c*-551i (Husain & Davidson, 1986), despite the fact that the measured E_m values indicated that electron transfer from reduced amicyanin to cytochrome *c*-551i was thermodynamically unfavorable. It was subsequently shown that amicyanin and methylamine dehydrogenase formed a weakly associated complex, which caused a large shift in the E_m value of amicyanin to +221 mV at low ionic strength (Gray et al., 1988). This complex-dependent shift in the E_m value of amicyanin is critical to the physiological function of these proteins as it significantly narrows the difference between the E_m values of amicyanin and cytochrome *c*-551i, thus facilitating what would be an otherwise thermodynamically unfavorable electron transfer. This complex-dependent shift of redox potential was essentially reversed by the addition of 0.2 M NaCl. High ionic strength was also shown to reverse complex-dependent perturbations of the absorbance spectrum of the protein-bound PQQ. These observations confirmed that methylamine dehydrogenase and amicyanin from *P. denitrificans* formed a physiologically relevant complex and suggested that it was stabilized by electrostatic interactions.

To better understand the specific interactions between methylamine dehydrogenase and amicyanin, a cross-linking study of these two proteins has been performed using the zero-length chemical cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). This cross-linker has been successfully used in studies of the interactions between small redox proteins that form electrostatically stabilized complexes (Davis & Hough, 1983; Vieira et al., 1986; Wynn & Malkin, 1988; Morand et al., 1989; Takabe & Ishikawa, 1989). EDC specifically modifies carboxyl groups located in surface domains of high negative charge density (Millet et al., 1983). The proteins are covalently cross-linked, without the introduction of a foreign spacer arm, by the formation of an amide linkage between the modified carboxylic acid on one protein and a positively charged amino group on another that is in close proximity. This study has allowed us to identify and characterize specific intramolecular interactions between subunits of methylamine dehydrogenase and intermolecular interactions between these subunits and amicyanin.

Experimental Procedures

Amicyanin and methylamine dehydrogenase were purified from *P. denitrificans* (ATCC 13543) as described previously (Husain & Davidson, 1985, 1987). The concentrations of these proteins were calculated from previously determined extinction coefficients (Husain & Davidson, 1985; Husain et al., 1987). Antibodies to amicyanin were elicited from female New Zealand White rabbits which were immunized subcutaneously at 2-week intervals, with 1 mg of pure protein, until a sufficiently high titer of antibodies was attained. EDC was purchased from Pierce.

In cross-linking experiments, proteins were incubated in 10 mM potassium phosphate buffer, pH 6.5 or 7.5, with freshly prepared EDC for 2 h at 25 °C. The choice of EDC concentration and time of incubation was based on the results of several trial experiments to determine the range of conditions that would optimize the formation of cross-linked species that resulted from specific protein–protein interactions. At concentrations of EDC higher that 5 mM and with longer incubation, nonspecific interactions as evidenced by the appearance of multiple high molecular weight species interfered with this analysis. Protein and EDC concentrations are given in the figure legends. To determine the effect of ionic strength on the complex formation, 0.2 M NaCl was included in the above reaction mixtures. Cross-linking experiments were terminated by dilution of samples into the sample buffer used for subsequent polyacrylamide gel electrophoresis (PAGE).

Nondenaturing discontinuous PAGE was performed essentially as described in the Hoeffer Scientific Instruments (San Francisco, CA) catalogue. The separating gel contained 9.5% acrylamide and 0.5% Bis in 0.24 M Tris-HCl, pH 8.5. The stacking gel contained 2.5% acrylamide and 0.6% Bis in 49 mM Tris–phosphate, pH 6.9. The cathode (top) buffer was composed of 37.6 mM Tris and 40 mM glycine, pH 8.9. The anode (bottom) buffer was composed of 63 mM Tris and 50 mM HCl, pH 7.5. The sample buffer contained 50% glycerol and 0.1% bromophenol blue. Electrophoresis was performed in slab gels with a minigel apparatus at a constant current of 10 mA until the tracking dye was 1.0 cm from the bottom of the gel. SDS–PAGE was performed by the method of Laemmli and Favre (1973) except for the inclusion of 0.5 M urea in the resolving and stacking gel and 4 M urea and 4% SDS in the final sample buffer. Western blotting with alkaline phosphatase conjugated IgG as a secondary antibody was performed with Bio-Rad reagents and equipment according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, CA). Molecular weights were determined by comparison with Bio-Rad prestained low molecular weight standards.

Methylamine dehydrogenase activity was measured spectrophotometrically with a dye-linked assay in which the oxidation of methylamine was coupled to a change in absorbance of a redox-sensitive dye, 2,6-dichlorophenolindophenol (Davidson, 1989). The ability of methylamine dehydrogenase and amicyanin to catalyze the methylamine-dependent reduction of cytochrome *c*-551i was assayed as described previously (Husain & Davidson, 1986).

Results

Nondenaturing PAGE Analysis of EDC-Treated Proteins. Since methylamine dehydrogenase is an oligomeric protein composed of four subunits, it was first necessary to examine the effects of EDC on methylamine dehydrogenase alone before studying its effects on mixtures of proteins. After incubation of methylamine dehydrogenase with 5 mM EDC, at either pH 6.5 or 7.5, samples were subjected to nondenaturing PAGE. Only a single band identical with that obtained with untreated methylamine dehydrogenase was observed (Figure 1). Thus, treatment of methylamine dehydrogenase alone with EDC did not cause formation of higher molecular weight aggregates or any change in mobility during nondenaturing PAGE. When methylamine dehydrogenase and amicyanin were incubated under identical conditions, the generation of two additional bands was clearly observed on nondenaturing PAGE (Figure 2). As methylamine de-

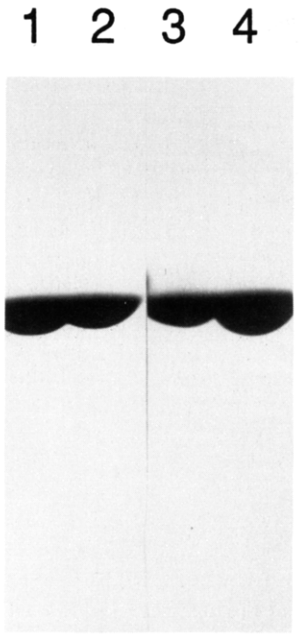

FIGURE 1: Nondenaturing PAGE analysis of EDC-treated methylamine dehydrogenase. Samples containing 8 μM methylamine dehydrogenase were incubated at pH 6.5 in the absence (lane 1) and presence (lane 2) of 5 mM EDC and at pH 7.5 in the absence (lane 3) and presence (lane 4) of 5 mM EDC. Gels were stained with Coomassie Blue R-250.

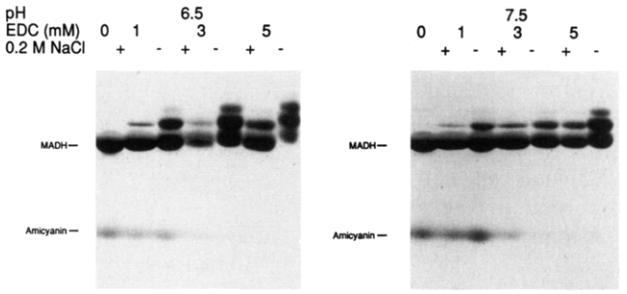

FIGURE 2: Nondenaturing PAGE of EDC-treated mixtures of methylamine dehydrogenase and amicyanin. Samples containing 8 μM methylamine dehydrogenase and 16 μM amicyanin were incubated as indicated at pH 6.5 or 7.5, in the presence of 0, 1, 3, or 5 mM EDC and in the presence or absence of 0.2 M NaCl. Gels were stained with Coomassie Blue R-250. The positions of the migration of untreated methylamine dehydrogenase (MADH) and amicyanin are indicated on the left of the gels.

hydrogenase alone exhibited no change in mobility when incubated in the absence of amicyanin, these two bands must represent cross-linked species that contain both methylamine dehydrogenase and amicyanin. It should be noted that the staining intensity of amicyanin is much less than that of methylamine dehydrogenase and, therefore, one cannot infer the molar ratio of these species from this figure. The two cross-linked species were generated during incubations performed at both pH 6.5 and 7.5. The cross-linked product appearing immediately above methylamine dehydrogenase was more readily formed at lower EDC concentrations. The second cross-linked product, which migrated just above the first, was only seen at higher EDC concentrations and was formed more readily at pH 6.5 than at pH 7.5. The generation of each species was dependent on ionic strength, since the staining intensities in the presence of added salt were lower compared to the corresponding lane in the absence of salt for a given EDC concentration. The formation of the second cross-linked species appeared to be more sensitive to ionic strength than the first. For each species, the effects of added salt were much more pronounced at pH 6.5 than at pH 7.5.

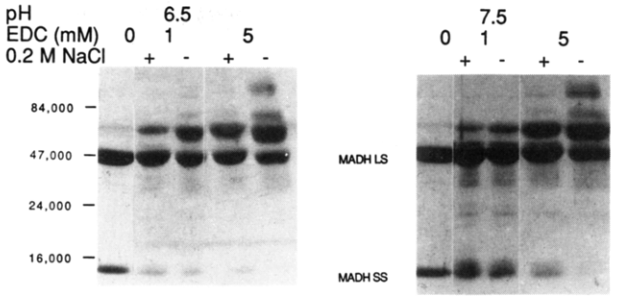

FIGURE 3: SDS–PAGE analysis of EDC-treated methylamine dehydrogenase. Samples containing 8 μM methylamine dehydrogenase were incubated as indicated at pH 6.5 or 7.5, in the presence of 0, 1, or 5 mM EDC and in the presence or absence of 0.2 M NaCl. The amount of sample loaded on the gels from the 5 mM incubations was twice that loaded for the other incubations. Samples were separated on a 12.5% polyacrylamide gel. Gels were stained with Coomassie Blue R-250. The positions of migration of molecular weight standards are indicated on the left. The positions of migration of the large subunit (LS) and small subunit (SS) of methylamine dehydrogenase (MADH) are indicated in between the two panels.

SDS–PAGE Analysis of EDC-Treated Proteins. To obtain more detailed information on the nature of the cross-linked species observed on nondenaturing PAGE, similar samples were denatured and subjected to SDS–PAGE and Western blot analysis. Again it was necessary first to examine the effects of EDC on methylamine dehydrogenase alone. While no intermolecular cross-linking was observed on nondenaturing PAGE, significant intramolecular interaction was evident from the analysis of denatured samples by SDS–PAGE (Figure 3). Under these conditions, the most prominent cross-linked product that was formed migrated with a M_r of about 61 000. This corresponds approximately to the combined molecular weights of one large and one small subunit of methylamine dehydrogenase and presumably represents a cross-linked species containing one of each of these subunits. The formation of this cross-linked product was not dependent on ionic strength or pH (Figure 3). At the highest EDC concentration, in the absence of salt, two additional minor bands were observed at each pH value. The molecular weights of these species suggest that they represent cross-linked combinations of one large plus two small subunits and two large plus two small subunits. The smaller subunits of methylamine dehydrogenase stained with much less intensity than the large subunit, and consequently it is difficult to monitor their disappearance. No evidence for the formation of a cross-linked species corresponding in size to two small subunits was ever observed. Furthermore, incubation of amicyanin alone with up to 5 mM EDC resulted in no appearance of higher molecular weight species (data not shown).

Analysis by SDS–PAGE of mixtures of methylamine dehydrogenase and amicyanin, which had been incubated with EDC under identical conditions, yielded results (Figure 4) that were very different than those described above for methylamine dehydrogenase alone. Again a cross-linked species of M_r approximately 61 000 was formed readily at low EDC concentration at either pH. At higher concentrations of EDC a second cross-linked species with a M_r of approximately 76 000 was observed, which was not evident when methylamine dehydrogenase alone was incubated with EDC. The formation this cross-linked species was much more dependent on ionic strength at pH 6.5 than at pH 7.5.

Western Blot Analysis of EDC-Treated Proteins. To determine which if any of these cross-linked species included amicyanin, these samples were subjected to SDS–PAGE followed by Western blot analysis with antibodies specific for

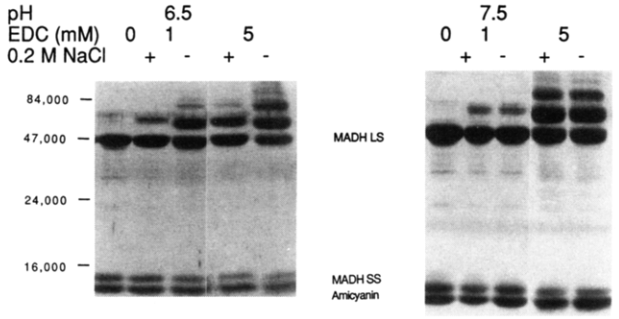

FIGURE 4: SDS–PAGE analysis of EDC-treated mixtures of methylamine dehydrogenase and amicyanin. Samples containing 4 μM methylamine dehydrogenase and 8 μM amicyanin were incubated as indicated at pH 6.5 or 7.5, in the presence of 0, 1, or 5 mM EDC and in the presence or absence of 0.2 M NaCl. The amount of sample loaded on the gels from the 5 mM incubations was twice that loaded for the other incubations. Samples were separated on a 12.5% polyacrylamide gel. Gels were stained with Coomassie Blue R-250. The positions of migration of molecular weight standards are indicated on the left. The positions of migration of amicyanin and the large subunit (LS) and small subunit (SS) of methylamine dehydrogenase (MADH) are indicated in between the two panels.

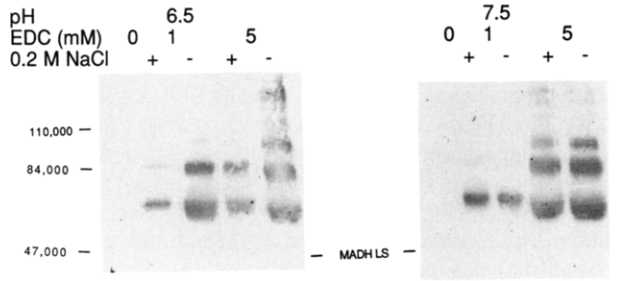

FIGURE 5: Western blot analysis of EDC-treated mixtures of methylamine dehydrogenase and amicyanin. Samples containing 1.3 μM methylamine dehydrogenase and 2.7 μM amicyanin were incubated as indicated at pH 6.5 or 7.5, in the presence of 0, 1, or 5 mM EDC and in the presence or absence of 0.2 M NaCl. Samples were separated on a 7.5% polyacrylamide gel and probed after electrophoretic transfer to nitrocellulose with antibody specific for amicyanin as described under Experimental Procedures. The positions of migration of molecular weight standards are indicated on the left. The position of migration of the large subunit of methylamine dehydrogenase (MADH LS), which does not stain in this procedure, is indicated in between the two panels.

amicyanin (Figure 5). For a better visualization of the cross-reacting cross-linked species, an immunoblot of a 7.5% gel is shown. On this percentage gel, amicyanin itself was not retained. The absence of a stained species in the control lane with no EDC illustrates the absolute lack of cross-reactivity of these antibodies with methylamine dehydrogenase. With the EDC-treated samples, cross-reactive species were observed, which migrated with M_r of approximately 61 000 and 76 000. The cross-linked species of M_r 61 000 observed in the Western blot must be a complex of one large subunit of methylamine dehydrogenase and one molecule of amicyanin. As the molecular weights of amicyanin and the small subunit of methylamine dehydrogenase are very similar, it was not possible to distinguish on a Coomassie-stained gel the cross-linked complexes that each of these could form with the large subunit. The band at M_r 61 000 observed in Figure 4 must contain both of these species (Table I). The cross-linked species of M_r 76 000 observed in the Western blot could, on the basis of molecular weights, correspond to a complex of either the large subunit with two molecules of amicyanin or the large subunit with one molecule each of amicyanin and the small subunit. It was not possible to probe these immunoblots for the presence of the small subunit, because, despite repeated efforts, we have been unable to raise antisera that specifically reacted with this subunit. Either of these putative species would also correspond to the band of M_r 76 000 on a Coomassie-stained gel in Figure 4 (Table I). At high EDC concentrations, a band with M_r of approximately 90 000 and minor higher molecular weight species are also observed in the Western blot. The appearance of each of the bands that were observed in the Western blot was strongly dependent on salt at pH 6.5, whereas little salt dependence is observed at pH 7.5.

Table I: Properties of Native Proteins and Observed and Putative Cross-Linked Species[a]

	M_r	cross-reactivity with antisera to amicyanin
native proteins		
amicyanin	15 000	+
MADH LS	46 700	–
MADH SS	15 500	–
observed cross-linked species		
species I (Figure 3)	61 000	–
species II (Figures 4 & 5)	61 000	+
species III (Figures 4 & 5)	76 000	+
putative cross-linked species		
MADH LS + MADH SS	62 200	–
MADH LS + amicyanin	61 700	+
MADH LS + MADH SS + amicyanin	77 200	+
MADH LS + 2 amicyanin	76 700	+

[a] Observed cross-linked species are the most prominent bands observed in SDS–PAGE and Western blot analysis. Putative cross-linked species are those that could have resulted from EDC treatment. M_r values of putative cross-linked species were calculated from known molecular weights of the native species. MADH, methylamine dehydrogenase; LS, large subunit; SS, small subunit.

Activity of EDC-Treated Proteins. To be sure that EDC treatment was not causing the formation, exclusively, of nonproductive cross-linked complexes, the catalytic and electron-transfer properties of the EDC-treated samples were assayed. Samples of methylamine dehydrogenase that had been incubated with 5 mM EDC at pH 6.5, similar to those shown in Figure 3, catalyzed the oxidation of methylamine at rates that were indistinguishable from those of untreated control samples. Samples of methylamine dehydrogenase plus amicyanin, which had been incubated with 5 mM EDC at pH 6.5, similar to those shown in Figure 2, were tested for their ability to catalyze the methylamine-dependent reduction of cytochrome *c*-551i. Those samples that exhibited a substantial degree of cross-linking on analysis by PAGE exhibited reaction rates that were approximately 63% of those of control samples that had not been exposed to EDC. Furthermore, no inhibition of activity was observed on preincubation with 1 mM EDC of the standard assay mixture of methylamine dehydrogenase, amicyanin, and cytochrome *c*-551i.

Discussion

The water-soluble carbodiimide EDC is well suited for studies of complex formation between proteins, which is stabilized by electrostatic interactions. It has been used previously to identify natural redox partners in biological systems (Wynn & Malkin, 1988) and to cross-link small redox proteins for the purpose of studying intermolecular electron transport (Davis & Hough, 1983; Vieira et al., 1986; Morand et al., 1989; Takabe & Ishikawa, 1989). In the present study it has been used to characterize the interaction between an oligomeric redox enzyme, methylamine dehydrogenase, and its physiological electron acceptor, amicyanin. Information on the intramolecular organization of methylamine dehydrogenase has been obtained as well. It is interesting to note that on incu-

bation of methylamine dehydrogenase alone with EDC no evidence for significant interactions between the two large subunits or between the two small subunits was observed. The principal intramolecular cross-linking that was observed was between a large subunit and a small subunit (Figure 2). These findings are in good agreement with a recent report of X-ray crystallographic studies of the methylamine dehydrogenase from *Thiobacillus versutus* (Vellieux et al., 1989). The crystal structure of that enzyme exhibited no direct interactions between the two small subunits, very limited interaction between the two large subunits, and extensive interaction between the large and small subunit. Thus, the data obtained in this study, particularly at lower EDC concentrations, compliment the existing crystallographic data and seem to accurately reflect the specific interactions occurring in this system.

The $\alpha_2\beta_2$ structure of methylamine dehydrogenase provides an opportunity to study in an oxidoreductase a subunit whose function is solely regulatory with no redox function of its own. Our results indicate that the primary site for amicyanin interaction is the large subunit of methylamine dehydrogenase. We have previously shown that the physical and antigenic properties of the small PQQ-bearing subunits of methylamine dehydrogenases are highly conserved over a wide range of taxonomically diverse bacteria (Davidson & Neher, 1986). The large subunits, however, exhibited significant variability. Immunological cross-reactivity was observed between the large subunits of enzymes from *P. denitrificans*, *T. versutus*, and *Pseudomonas* AM1 (Davidson & Neher, 1986). In each of these bacteria, the primary electron acceptor for methylamine dehydrogenase is amicyanin (Husain & Davidson, 1985; van Houwelingen et al., 1985; Tobari & Harada, 1981). No immunological cross-reactivity was observed between the large subunits of the enzymes from *P. denitrificans* and bacterium W3A1 (Davidson & Neher, 1986). Amicyanin has not been found in the latter organism, and it has been suggested that, instead, a *c*-type cytochrome is the electron acceptor (Chandrasekar & Klapper, 1986). Thus, the primary role of the large subunit of methylamine dehydrogenase may not relate to the catalytic reductive half-reaction with the amine substrate but to the oxidative half-reaction with its physiological electron acceptor.

An important aspect of this work is the further characterization of the dependence of complex formation on ionic strength. The results of these cross-linking studies suggest that complex formation involves at least two, and probably more, specific interactions. These interactions do not each exhibit the same dependence on ionic strength. Previous observations have suggested that attractive electrostatic interactions were responsible for orienting these two redox proteins in a manner that facilitated electron transfer. However, while it had been documented that the large complex-dependent shift in redox potential of amicyanin did not occur in high salt, electron-transport activity, albeit at greatly reduced levels, was still observed (Gray et al., 1988). Furthermore, the spectral perturbations caused by the interaction of the two proteins at low ionic strength were mostly, but not completely, eliminated on addition of salt (Gray et al., 1988). A simple interpretation of these data is as follows. It is clear that significant cross-linking of amicyanin with the large subunit can occur at low EDC concentrations even in the presence of added salt (Figure 5). Thus, amicyanin may bind to the large subunit in a manner that can be stabilized at least in part by hydrophobic interactions. This association is critical in that it serves to anchor these proteins in an orientation in which the prosthetic groups of amicyanin and the small subunit of methylamine dehydrogenase are in reasonable proximity to each other but not directly interacting. Under these conditions, amicyanin is cross-linked only to the larger subunit. Those groups on amicyanin and the small subunit that participate in intermolecular electron transfer then interact in a manner that is electrostatically stabilized. Under these conditions, amicyanin can be cross-linked to both the larger and smaller subunits. It is this interaction with the small subunit that results in the shift in redox potential of the copper center of amicyanin. The observation that low levels of amicyanin-mediated reduction of cytochrome *c*-551i persist in the presence of salt, despite a thermodynamically unfavorable difference in redox potentials, can be explained by proximity effects that result from the interaction of amicyanin with the large subunit.

This study demonstrates that amicyanin and methylamine dehydrogenase can be cross-linked with EDC and that this experimental system will allow study of the specific interactions of amicyanin with individual subunits of methylamine dehydrogenase and of factors that influence complex formation such as ionic strength and pH. It provides a basis for future investigation of the binding interface of the two partner proteins, and of the identity of specific amino acids that are involved in their physiologically relevant interactions. Another intriguing aspect of this work relates to the recent confirmation that several mammalian oxidoreductases possess both covalently bound PQQ and copper as cofactors in a single enzyme. These enzymes include bovine plasma amine oxidase (Lobenstein-Verbeek et al., 1984), kidney diamine oxidase (Paz et al., 1988), bovine adrenal dopamine β-hydroxylase (van der Meer et al., 1988), and human placental lysyl oxidase (van der Meer & Duine, 1986; Williamson et al., 1986). The mechanisms by which PQQ and copper interact in these enzymes are not at all understood. As such, continued study of the interaction between the quinoprotein methylamine dehydrogenase and the copper protein amicyanin will not only provide insight into the mechanisms of intermolecular electron transport and protein–protein interactions but also may provide information on the evolution and mechanisms of an important family of eukaryotic enzymes.

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An Unusual Specificity in the Activation of Neutrophil Serine Proteinase Zymogens†

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ABSTRACT: The majority of proteinases exist as zymogens whose activation usually results from a single proteolytic event. Two notable exceptions to this generalization are the serine proteinases neutrophil elastase (HNE) and cathepsin G (cat G), proteolytic enzymes of human neutrophils that are apparently fully active in their storage granules. On the basis of amino acid sequences inferred from the gene and cDNAs encoding these enzymes, it is likely that both are synthesized as precursors containing unusual C-terminal and N-terminal peptide extensions absent from the mature proteins. We have used biosynthetic radiolabeling and radiosequencing techniques to identify the kinetics of activation of both proteinases in the promonocyte-like cell line U937. We find that both N- and C-terminal extensions are removed about 90 min after the onset of synthesis, resulting in the activation of the proteinases. HNE and cat G are, therefore, transiently present as zymogens, presumably to protect the biosynthetic machinery of the cell from adventitious proteolysis. Activation results from cleavage following a glutamic acid residue to give an activation specificity opposite to those of almost all other serine proteinase zymogens, but shared, possibly, by the "granzyme" group of related serine proteinases present in the killer granules of cytotoxic T-lymphocytes and rat mast cell proteinase II.

Most proteolytic enzymes are stored as inactive precursors (zymogens), presumably to protect the biosynthetic and transport machinery of the body from adventitious proteolysis. Upon reaching their target locations, zymogens await activation that occurs following limited proteolysis by specific activator proteases. This process, reviewed recently by Neurath (1989), occurs in most members of the chymotrypsin superfamily of serine proteinases, the most numerous proteinase superfamily, whose members include the coagulation, fibrinolytic, and pancreatic proteinases.

The specificity and chemical principles of zymogen activation are best understood for the pancreatic proteinases chymotrypsin and trypsin. A single proteolytic event (cleavage of the Arg-15/Ile-16 bond in chymotrypsinogen or the equivalent Lys-6/Ile-7 bond in trypsinogen) generates an α-amino group in a location that enables the proteins to adopt their catalytic conformations (Kraut, 1977). Other cleavages may occur (Miller et al., 1971), but these do not result in zymogen activation. Whereas the structural changes associated with zymogen activation of chymotrypsin superfamily members are only known for the pancreatic enzymes, the specificity of activation is conserved for almost all members, with activation following cleavage after a basic amino acid (Lys or Arg).

The neutrophil proteinases elastase (HNE)[1] and cathepsin G (cat G), members of the chymotrypsin superfamily (Sinha et al., 1987; Salvesen et al., 1987), exist as catalytically competent forms in their storage granule (Starkey, 1978; Senior

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[1] Abbreviations: HNE, human neutrophil elastase (EC 3.4.21.37); cat G, cathepsin G (EC 3.4.21.20); E-64, *N*-[4-[[*N*-[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]amino]butyl]guanidine; DCI, 3,4-dichloroisocoumarin; ATZ, anilinothiazolinone; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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