The Covalent Structure of the Small Subunit from *Pseudomonas putida* Amine Dehydrogenase Reveals the Presence of Three Novel Types of Internal Cross-linkages, All Involving Cysteine in a Thioether Bond*

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*Pseudomonas putida* contains an amine dehydrogenase that is called a quinohemoprotein as it contains a quinone and two hemes as redox active groups. Amino acid sequence analysis of the smallest (8.5 kDa), quinone-cofactor-bearing subunit of this heterotrimeric enzyme encountered difficulties in the interpretation of the results at several sites of the polypeptide chain. As this suggested posttranslational modifications of the subunit, the structural genes for this enzyme were determined and mass spectrometric de novo sequencing was applied to several peptides obtained by chemical or enzymatic cleavage. In agreement with the interpretation of the X-ray electronic densities in the diffraction data for the holoenzyme, our results show that the polypeptide of the small subunit contains four intrachain cross-linkages in which the sulfur atom of a cysteine residue is involved. Two of these cross-linkages occur with the β-carbon atom of an aspartic acid, one with the γ-carbon atom of a glutamic acid and the fourth with a tryptophanquinone residue, this adduct constituting the enzyme’s quinone cofactor, CTQ. The thioether type bond in all four of these adducts has never been found in other proteins. CTQ is a novel cofactor in the series of the recently discovered quinone cofactors found in the types established so far (1). Based on the natural electron acceptor used, a further distinction can be made between amine oxidases and amine dehydrogenases. Both classes convert amines into their corresponding aldehydes, but oxidases produce toxic peroxides, while the reducing equivalents in the case of dehydrogenases are directly transferred to the respiratory chain (2).

Depending on the identity of their cofactor(s), amine dehydrogenases are subdivided into quinoproteins, flavoproteins, quinohemoproteins, and flavohemoproteins (2). *Pseudomonas putida* strain ATCC 12633, as well as strain IFO 15633, contain a novel type of amine dehydrogenase; a quinohemoprotein (QH-AmDH) as a quinone compound is present in the small subunit and two heme c groups in the large subunit (3). Although the quinone cofactor was not liberated on denaturing the enzyme, spectroscopic data (4) already indicated that it is different from tryptophan tryptophylquinone (5), topaquinone (6), or lysine tyrosylquinone (7), forming part of the protein chain of several amine dehydrogenases (EC 1.4.99.3/4), several amine oxidases (EC 1.4.3.6), and protein-lysine 6-oxidase (EC 1.4.3.13), respectively.

To reveal the identity of the quinone cofactor and its position in the protein, the genes for QH-AmDH were cloned and sequenced, and the small subunit subjected to chemical analysis. The latter was carried out in a combination of automated Edman degradation, mass spectrometry, liquid chromatography, and fragmentation MS applied to the underivatized and the derivatized form (the quinone cofactor converted into a hydrazone with a hydrazine) of the small subunit, as well as to several peptides obtained by chemical or enzymatic cleavage. Interpretation of the results was facilitated by the recently obtained progress in elucidating the crystal structure of the enzyme.

A diversity of enzymes appears to be involved in amine oxidation, as reflected by the number of different cofactors.

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1 The abbreviations used are: QH-AmDH, quinohemoprotein amine dehydrogenase; MS, mass spectrometry; Mops, 4-morpholinepropane-sulfonic acid; CDAP, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate; TOF, time-of-flight; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; kbp, kilobase pair(s); ESI, electrospray ionization; ORF, open reading frame; CTQ, cysteinyl tryptophanquinone.

2 A Satoh, J.-K. Kim, I. Miyahara, B. Devreese, I. Vandenbergha, A. Hacisalihoglu, T. Okajima, S. Kuroda, O. Adachi, J. A. Duine, J. Van Beeumen, K. Tanizawa, and K. Hirotsu, unpublished data.
Experimental Procedures

Isolation and Purification of the Small Subunit—The small subunit of QH-AmDH from strain ATCC 15363 was isolated as described in (4). The enzyme preparation was brought to 8 M urea and to pH 8.5 with 1 M NaOH. The mixture was heated for 5 min at 95 °C, cooled, and centrifuged to remove the precipitate, and the supernatants were brought onto a Superdex 75 column equilibrated with 50 mM sodium phosphate buffer, pH 8.5, containing 8 M urea. Elution occurred with the same buffer, enabling the separation of the small subunit from the other two subunits as monitored by a Hewlett-Packard 1040 photodiode array detector. Urea was removed from the small subunit-containing fraction by loading it onto a Pharmacia Superdex peptide column equilibrated with 20 mM Mops buffer, pH 7.0. The preparation was desalted over Centricon devices with a cut-off of 3 kDa (Millipore Corporation, Bedford, MA). The small subunit of the QH-AmDH from strain IFO 15633 was isolated as described in (3). The high amount of sucrose used as a stabilizing agent was eliminated, and the sample was concentrated by ultrafiltration using Vivaspin centrifugal devices with a 5 kDa cut-off (Vivasin, Binbrook, UK). After a wash with 500 μl of water, the concentrated sample was incubated overnight with the buffer 6 M guanidine/0.5 M Tris-HCl, pH 8.5, at room temperature to dissociate the subunits. The small subunit was separated from the α and β subunits by two successive gel filtration experiments on a Superdex X75 PC 3.2/30 column (Amersham Pharmacia Biotech) and eluted by SMART separation system (Amersham Pharmacia Biotech) using the before mentioned buffer as eluent. To reduce the salt concentration during gel filtration, we switched to 1.2 M guanidine/0.1 M Tris-HCl, pH 8, as elution buffer. The desalting of the fraction containing the small subunit was performed over Vivaspin centrifugal devices.

Sequence and Mass Spectrometric Analyses—Internal peptides of the small subunit were obtained by chemical and enzymatic cleavages. Partial acid hydrolysis was performed on the intact polypeptide from strain ATCC 12633. Seven nanomoles were incubated for 2 h at 106 °C in 2% formic acid. Enzymatically obtained peptides (strain IFO 15366) were subcleaved by partial acid hydrolysis for 3 h in 0.22% HCl at 99 °C in 2% formic acid. Enzymatically obtained peptides (strain ATCC 12633) were treated with bromopropylamine under denaturing circumstances (8). Another modification experiment was attempted using CDAP to cyanate potential cysteines of the protein of strain IFO 15366 prior to cleavage with 2M ammonium hydroxide (9). The reaction mixture was desalted by ultrafiltration, using Vivaspin (Vivasin, Binbrook, UK) centrifugal devices with 5 kDa cut-off. Chemical sequencing of the small subunit and its internal peptides was performed on a 476A pulsed liquid sequenator equipped with an on-line phenylthiohydantoin-derivative analyzer (Applied Biosystems, Foster City, CA).

Mass determinations from protein and peptides were done on a nano-electrospray ionization hybrid-quadrupole TOF mass spectrometer (Micromass, Wythenshawe, UK). Fragmentation spectra for de novo sequencing aims were established with the same instrument. The liquid chromatography mass spectrometry configuration, used for experiments on peptide mixtures resulting from the digestions with Pronase and Glu-C endoproteinase, is the same as described in (10). The masses of most of the peptides were measured on a TofSpec SE TOF instrument (MALDI-TOF-MS) equipped with a nitrogen-laser (337 nm) (Micromass, Wythenshawe, UK). Scans were accumulated over 20–70 laser shots, using urea-cyanohydronic acid as matrix. The instrument was calibrated externally prior to analyses using both angiotensin II and bovine insulin (Sigma).

Cloning and Sequence Analysis of the Gene Encoding QH-AmDH from Strain IFO 15366—Two degenerate oligonucleotide primers were designed based on the N-terminal 9-residue sequence of the β subunit obtained after electrophoretic blotting of the SDS-PAGE (14% acrylamide)-separated subunits of the enzyme (9) and based on an internal 7-residue sequence of the same subunit obtained by endoproteinase Lys-C digestion of the enzyme. Primer P1 (sense strand, 23-mer) reads: 5′-GA(C/T)ACGIGIICCIITTTAIA/GC/5′, where I is inosine. Primer P2 (antisense strand, 21-mer) reads: 5′-CATIAG/TTCICCCIGIGGIA/GC/5′. The primers were used for amplification of a part of the gene by polymerase chain reaction with the Ps. putida genomic DNA as a template. The mixture (100 μl) for the polymerase chain reaction, processed on a thermal cycler (PerkinElmer Life Sciences), contained 300 ng of the template DNA, 0.5 μM of each primer, 200 μM of each dNTP, and 1 unit of Taq DNA polymerase. The initial denaturation step at 94 °C for 30 s was followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extending at 68 °C for 2 min. The amplified fragment of about 1.0 kbp was subcloned into a pT7Blue-T

Fig. 1. Electrospray mass spectrum of the small subunit of Ps. putida amine dehydrogenase, strain ATCC 12633. The original multiple charged spectrum is transformed using MaxEnt.
vector (Novagen) for DNA sequence determination with a Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA), radiolabeled with \[^32P\]-dCTP (6000 Ci/mmol), and then used as a probe for Southern hybridization of the BglII- or PstI-digested genomic DNA. Genomic DNA libraries were constructed by inserting BglII- or PstI-fragments (4–6 kbp), positively hybridized with the probe, into the vector pUC19 (Takara, Kyoto, Japan) and transformed into *Escherichia coli* DH5 cells to give 10^6-10^7 clones. Colony hybridization using the same probe led to isolation of clones containing a BglII (about 6 kbp) or PstI (about 5 kbp) fragment. Restriction analysis indicated that the two fragments overlapped each other, covering the entire gene for the enzyme of about 5 kbp. DNA sequence analyses were done in both directions of the two fragments using oligonucleotide primers synthesized on the basis of the farthest region of the preceding sequencing.

**RESULTS AND DISCUSSION**

**Protein Chemical Analyses**—The electrospray mass spectrum of the small subunit of QH-AmDH from strain ATCC 12633 gave molecular masses of 8486.5 Da and 8504.0 Da (Fig. 1). Apparently, the latter mass was due to an oxidized form since it disappeared upon dithiothreitol treatment (data not shown). N-terminal sequence analysis of the polypeptide revealed the identity of 31 of the first 35 residues, with gaps of information at positions 6, 15, 26, and 32 (Fig. 2). A noticeable feature in this sequence was the occurrence of a tryptophan residue at position 14, which therefore was unlikely to form part of the quinone cofactor structure. It should be mentioned here that the protein begins at residue 2 of the gene sequence (see below) and, therefore, that the numbering for the protein sequence differs from that of the gene sequence by 1 residue.

It should be noted in this context that the molecular mass of the small subunit as determined with SDS-PAGE calibrated with the common reference proteins, has been reported to be 20 kDa (3). However, the value of 8.5 kDa found here is in line with that deduced from the gene for the small subunit (see below). It seems, therefore, that the high value obtained with SDS-PAGE must be due to anomalous behavior of the small subunit caused by a deviating shape induced by the four cross linkages (see below).

To generate peptides, two enzymatic cleavages, one with endoproteinase Lys-C and one with chymotrypsin, were performed as well as a chemical cleavage using dilute acid. Endoproteinase Lys-C did not generate fragments significantly different from that given by the native polypeptide chain. Both the other enzymatic digest and the partial hydrolysis yielded peptides that corroborated the N-terminal sequence data as well as revealed the C-terminal sequence of the protein, covering the region Met-49-Lys-78. Mass analysis data that confirmed the assignments for the latter region are given in Table I. At this stage, no peptides could be found that linked the N-terminal segment Met-49-Asp-55 and the beginning of the C-terminal part of the protein. Sequencing of the peptide CNBr 26, obtained by CNBr cleavage of the polypeptide chain, did reveal the identity of residues Asp-38, Pro-39, and Trp-41, but no sequence information was obtained from residue 42 onwards. Based on the detection of an Asp-Pro bond at positions 9 and 10 of the peptide, a cleavage specific for this acid labile bond was carried out on the native subunit. Edman degradation of one of the resulting Asp-Pro (DP) peptides finally did provide the overlap between the N-terminal and the C-terminal parts of the protein (Fig. 2).
The completion of the sequence of the subunit was obtained by aid of mass analysis data for the individual peptides, as well as from MS-MS de novo sequencing data on those peptides where Edman degradation failed to identify a residue at some particular positions. Because some of these positions were initially suspected to be taken by cysteines, the polypeptide was initially reduced and treated with bromopropylamine. Mass spectrometric analysis showed, however, that the molecular mass of the protein remained unchanged.

Intrachain Linkages between a Cysteine Residue and a Methylenic Group of an Acidic Residue—During the course of the chemical sequencing work, the gene sequence of QH-AmDH from strain IFO 15366 (but not from strain ATCC 12633) has been determined (see below). Very recently, the results of the nearly completed x-ray structural analysis of the enzyme became available to us.\(^3\) It shows electron densities revealing the existence of four internal cross-linkages between the residues 6 and 15, 26 and 32, 36 and 42, and 40 and 48. By combining two observations, one from the Edman degradation of peptide AH38 and one from the electrospray mass analysis of this peptide, we can state that the cross-link between the residues 6 and 15 is made between the side chains of a cysteine residue and a methylenic carbon atom of a glutamic acid residue, forming a thioether bond. Indeed, the molecular mass of the peptide was measured to be 15.2 Da higher than the theoretical mass of 1564.7 Da for the non-cross-linked peptide. The difference is in agreement with the existence of the thioether linkage between Cys-6 and Glu-15 (\(\Delta m = 2\) Da) in combination with the fact that the acid labile peptide bond Asp-11-Pro-12 was cleaved (\(+18\) Da) and that Asp-11 has been removed as well during the hydrolysis procedure to generate the peptide. Such a cleavage also explains why, along with the N-terminal SAVAG-sequence, a sequence PGWXV (X unidentified) was also found with a roughly similar yield. The conclusions are also confirmed by MS-MS analysis of peptide AH38, the result of which is given in Fig. 3. The spectrum contains two b- and two y-series: b, y, and y, corresponding to the two cross-linked peptides. Information on the N-terminal sequence of the first peptide is interrupted at the b4/y6 fragment. A mass increment of 802.8 Da at position b5 in the b-ion series is due to the formation of the fragment SAVAGC linked with peptide PGWEVD, demonstrating a covalent linkage between the two peptides. The rest of the series is then formed by a normal sequential fragmentation pattern corresponding to TATT. Also y-ions are observed, corresponding to the fragments containing DTTATC linked with PGWEVD, were found. The second series starts its N-terminal fragmentation at Pro-12, the y series starts at Asp-17.

The second thioether cross-link, between Cys-26 and, in this case, an aspartic acid side chain (residue 32) was proven from the MS-MS analysis of peptide Ala-18-Asp-32. The peptide was obtained as the result of a subcleavage, by partial hydrolysis, of a larger peptide, Ala-18-Gln-54, which in fact contains three of the four protein cross-links (Fig. 2) and which itself was obtained by cleavage of the subunit with N-Asp protease. The experimental mass of the former peptide was 1569.4 Da, which is exactly 2 mass units smaller than the theoretical mass without cross-link. The thioether bond occurs with the sulfur atom and the methylene carbon atom of the aspartic acid. The MS fragmentation spectrum (result not shown) displays sequence information for the first 8 residues of the peptide, both via b- and y-fragments, but does not reveal any information on the last 7 residues where the cross-link occurs between the first and the last residue. We also detected an oxidized form of this peptide. MS-MS data clearly show that the oxidation is situated in the peptide Cys-26-Asp-32. We assign the oxidation to the methionine at position 29, since this residue is the only plausible candidate for oxidation. This may be the origin of the oxidized species we observed during ESI-MS analysis of the entire subunit (Fig. 1). With respect to enzyme specificity, it should be noted that the N-Asp protease was not able to cleave

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\(^3\) A. Satoh, personal communication.
ahead the cross-linked aspartic acid. Partial acid hydrolysis, however, did cleave the peptide bond between the cross-linked aspartate and the subsequent residue (Leu-33) but did not cleave the thioether link.

**Intrachain Linkage between Cysteine and Tryptophanquinone**—The two remaining internal cross-links could not be separately demonstrated because two of the residues involved, Cys-40 and Trp-42, are sequentially located very close to each other and, individually, take part in different linkages. The peptide including both a Cys-Trp and a Cys-Asp cross-linkage was obtained from the Pronase digest of the subunit and is labeled as "Pron 6" in Fig. 2. Its mass of 1862.9 Da corresponds to the calculated value of 1837.1 Da for the sequence Leu-33-Asp-48, augmented with 25.8 Da. The increase is as expected, since it combines an increment of 28 Da due to the cofactor cross-link with a loss of 2 Da due to the thioether linkage (H2Da).

**Analysis of Peptides Containing the Central Three and All Four Cross-linkages**—Treatment of the native subunit of the ATCC enzyme with chymotrypsin allowed isolating the 34-residue peptide Gly-20-Tyr-53 (named CH 19), which contains three of the four cross-linkages (Fig. 2). Whereas Edman degradation confirmed the residues that were already found by N-terminal analysis of the native protein, MS-MS analysis also allowed to determine the last 5 residues. The experimentally determined mass for this peptide, analyzed by MALDI-TOF MS, is 3750 Da, which indicates an additional value of 24 Da in comparison to the calculated mass for the unmodified form. This difference is consistent with the mass increment that theoretically should be expected for the presence of the cysteinyl tryptophanquinone cross-link (supplement of +28 Da) and the two thioether cross-linkages (+4 Da) in this peptide. Fragmentation was performed on the triply charged ion with m/z = 1251 Da observed in the ESI-MS spectrum. Typical for the fragmentation spectrum (Fig. 4) is the information stop at Leu-25 in the N-terminal b-ion series and at Met-49 in the C-terminal y-ion series, with exception of the small region between the residues Asp-32 and Cys-36, which are themselves cross-linked respectively to Cys-26 and to the tryptophanquinone at position 42. This can be explained by low or no fragmentation of the peptide within the two cross-linking residues. Assuming this, we may expect a mass increment of 772.9 Da in the ion patterns due to the thioether linkages between Cys-26 and Asp-32. This increment is indeed found in both the b-series as well as in the y-series. Another gap in the fragmentation spectrum, due to the quinone cross-link in combination with the thioether link Cys-40-Asp-48, was found in both the N-terminal and C-terminal ion series (Fig. 4). As a final proof for the occurrence of the four cross-linkages, we also isolated, from the chymotryptic digestion product of the subunit of strain ATCC 12633, a quadruply charged mass ion with m/z = 1408.0 Da, corresponding to a molecular weight of 5628 Da. By fragmentation of this ion, the corresponding peptide was characterized as Ser-1-Tyr-53, a peptide that also includes the cofactor (CH 20 in Fig. 2). The mass difference between the experimental and the calculated masses equals 22 Da, which is consistent with the expected mass aberration as a consequence of the peculiar covalent structure of this polypeptide.

The remarkable covalent structure of the small subunit of the amine dehydrogenase now allows us to understand why enzymatic proteolytic cleavages failed to proceed in the region 20–53 despite the presence of several peptide bonds, which under standard conditions would have been easily cleaved e.g. by chymotrypsin. The structure also explains why the CNBr fractions also showed molecular masses that were far beyond the theoretical masses. In fact, the peptides were all linked to each other. The three types of internal cross-linkages are schematically drawn in Fig. 5.

**Discrepancy between Masses Measured by MALDI-TOF-MS and ESI-MS**—A noticeable feature in this study is the fact that the molecular weight of all the peptides and peptide fragment ions containing the tryptophanquinone cofactor were 1 Da smaller than expected for the (multiple) protonated species. This systematic discrepancy can be explained when we assume that a radical cation at this quinone is formed during the
electrospray ionization process, adding a charge without an extra mass, in contrast to the protonation that occurs during standard ion formation by this type of ionization. An example is peptide CH 19, for which the triple charged fragment ion has an m/z = 1250.3, corresponding to 3747.9 Da after conversion, whereas the value obtained by MALDI-TOF-MS is 3749 Da.

The Enzyme from Ps. putida IFO 15366—The enzyme from strain IFO 15366 had become available shortly after that of strain ATCC 12633. The former was taken through many similar but also some different procedures from those applied to the enzyme of strain ATCC 12633 (see "Experimental Procedures"). Initially the protein was treated with a cysteine-cyanating reagent, namely CDAP, followed by cleavage in 2 M ammonia. No peptides were obtained and, as for the protein from strain ATCC 12633, no chemically modified cysteine could be detected, pointing to the fact that both polypeptide chains do not contain a free cysteine residue or any reducible disulfide bridge. Furthermore, we obtained the same type of peptides using the same protease for major digests or subdigests as for the strain ATCC 12633 enzyme. The only difference was found in the peptide CH 15 a (Fig. 2), where a serine occurs instead of a threonine at position 61 and a glutamate was found instead of an aspartic acid at position 65, in agreement with the gene sequence described below. The value of the masses of the two amino acids in each sequence turns out to be the same (216 Da). Since we found no other mutual replacements in the two enzymes, the overall mass of the enzyme from strain IFO 15366 is the same as that of strain ATCC 12633, namely 8488 Da (spectrum not shown).

For the sake of completeness, it may be mentioned that the liquid chromatography mass spectrometry spectra from a Pronase digestion and a Glu-C protease digestion of the subunit from the enzyme of strain IFO 15366 showed several N-terminal peptides, all ending at Asp-17 and beginning at either Ser-1, Ala-2 or Val-3, for which the experimental masses were 2 Da less than their calculated values. These mass results confirm the thioether cross-link Cys-6-Glu-15. The evidence for the other three thioether cross-linkages, including the one that

**FIG. 4.** MS/MS analysis of the chymotryptic peptide CH19. See also the legend to Fig. 3.

**FIG. 5.** The structures of the three novel thioether containing internal cross-linkages in the *Ps. putida* amine dehydrogenases. *a,* represents the new quinone cofactor, CTQ; *b* and *c* show the links between cysteine and the side chains of the acidic residues Asp and Glu.
involves the tryptophanquinone cofactor, as is for the enzyme of strain ATCC 12633. Several mass results for these peptides indicate the presence of an oxidation product, presumably at Thr-14.

Gene Structure of the Enzyme from Ps. putida IFO 15366—
Structural genes coding for the three subunits of the enzyme were cloned in two overlapping restriction fragments from the Ps. putida genome. There are four open reading frames (ORF) in the region of about 5000 nucleotides in length (Fig. 6). Because Edman sequencing of the α subunit after SDS-PAGE of the purified enzyme yielded the N-terminal sequence EQGPSLLQN-, and because an NTG codon (N is either A, G, C, or T) with a spacing of 7–8 bases 3’-downstream from the putative ribosome-binding site (5’-GGAGG-3’) is sometimes read as the translational initiation Met codon in Prostasoma genes (12), the 27-residue sequence MKTTRLRRHAGKLALVAAALLSTQAMA (see Fig. 7) is likely to be a signal peptide directing the translocation of the enzyme into the periplasm (3). Thus, the α subunit is encoded in the first ORF consisting of 1563 nucleotides (521 amino acids); the mature α subunit consists of 494 residues with a calculated molecular mass of 53,917 Da. The deduced primary structure of the α subunit contains two consensus sequences, CXXC (X is an unspecified residue), for the binding of heme groups consistent with the heme c content in the α subunit (3). The β subunit is encoded in the fourth ORF of 1119 nucleotides (373 residues), in which the 24-residue sequence MKAGRCASLALTIAAAACASHL from the presumed Met initiator similarly appears to function as a signal peptide; Edman sequencing of the purified β subunit indeed provided the sequence ADTGPALKA. Thus the mature β subunit contains 349 amino acid residues, and its calculated molecular mass is 39,234 Da. The gene for the γ subunit constitutes the third but small ORF (237 nucleotides), which is separated by 17 nucleotides 5’-upstream from the ORF for the β subunit; it encodes 79 amino acid residues, without a notable signal peptide. The calculated molecular mass of the γ subunit is 8,597 Da for the peptide lacking any modification. The amino acid sequence of the γ subunit deduced from the nucleotide sequence of its coding gene agrees perfectly with that determined chemically, as described above. A homology search of the protein sequences deposited in the GenBank™ protein sequence data base failed to detect any protein similar (with >30% identities) to either one of α, β, and γ subunit sequences.

The second ORF, consisting of 1455 nucleotides, does not correspond to any subunit of the enzyme. If the ATG codon, 48 nucleotides 3’-downstream from the termination codon of the α subunit gene, is taken as the translation initiation site, this ORF potentially encodes a protein of 476 residues with a calculated molecular mass of 53,160 Da. The deduced amino acid sequence of the second ORF shows an overall weak but locally significant similarity with the sequences reported for the AslB (formerly misnamed AtsB) gene product from Klebsiella pneumoniae and for some other related hypothetical proteins annotated via bacterial genome projects (Fig. 8). AslB is an iron sulfur protein that is required for the oxidation of a specific serine or cysteine residue in sulfatases to posttranslationally generate a formylglycine (2-oxoalanine) cofactor (13), hence designated as an arylsulfatase-activating enzyme. The Cys-rich region (CXXCXXXC, where X is an unspecified residue) predicted to serve as a binding motif for the [Fe-S]-cluster in AslB is highly conserved in the ORF 2 protein of Ps. putida (Fig. 8). Furthermore, AslB, as well as the activating enzymes for pyruvate formate-lyase and anaerobic ribonucleotide reductase, is included in a novel protein superfamily, named “Radical SAM Proteins” (3-adenosylmethionine), as identified by a recent bioinformatics search (14). Radical SAM proteins, sharing the Cys-rich [Fe-S]-binding motif and a Gly-rich sequence that is likely involved in binding SAM, catalyze diverse reactions, such as unusual methylations, isomerization, sulfur insertion, ring formation, anaerobic oxidation, and protein radical formation. The SAM binding Gly-rich sequence is also conserved in the ORF 2 protein of Ps. putida (Fig. 8). This ORF, intervening between the structural genes for α and γ subunits of the enzyme, thus encodes a new member of the radical SAM Proteins superfamily.

Considerations on the Biogenesis of the CTQ Cofactor and the Internal Cross-links—The γ subunit undergoes three types of posttranslational modifications during the maturation of the enzyme. Not necessarily in the given order, these modifications are 1) the formation of a cysteine to tryptophan cross-linkage, 2) the introduction of a pair of oxygen atoms into the tryptophan ring, and 3) the cross-linking of cysteine side chains to the
Cβ or Cγ atoms of carboxylic acid side chains. Any or all of these events can in principle occur by either an autocatalytic process or by the action of one or more external enzymes. Examples of an autocatalytic system are the biogenesis of the topaquinone cofactor in copper amine oxidases (15) and of the Cys-Tyr cofactor in galactose oxidase (16), in the latter case with the formation of a thioether linkage. An example of an enzymatic process is the biosynthesis of tryptophan tryptophanquinone in methylamine dehydrogenase, where the absence of one of the genes (mauG) of the 11 genes in the mau operon appears to prevent the maturation of tryptophan tryptophanquinone (17, 18).

We here propose that the biosynthetic process of the maturation of the CTQ cofactor in the γ subunit of Ps. putida amine dehydrogenase may involve an enzyme-mediated process as well. A likely candidate is the AslB-like [Fe-S] protein encoded in ORF 2 of the enzyme genes (Fig. 8). Similar to the Klebsiella AslB protein, which is essential for posttranslational oxidation of a serine or cysteine residue to formylglycine in sulfatase enzymes. The AslB protein, which is essential for posttranslational oxidation of a serine or cysteine residue to formylglycine in sulfatase enzymes, contains an unusual [Fe-S] center (19). In this respect, it is noteworthy that carbon-sulfur bond-forming enzymes (biotin and lipoate synthases) and peptidyl glycyl radical-forming enzymes (activating enzymes for anaerobic ribonucleotide reductase, pyruvate formate-lyase, and presumably benzylsuccinate synthase) all are radical SAM proteins utilizing an intermediate adenosyl radical in their catalytic processes. For all these reasons, we conclude that the biogenesis of the CTQ cofactor, as well as the highly unusual cross-linking structure within the γ subunit proceeds through a redox process, which most likely involves the presumed enzyme-activating radical SAM enzyme encoded in the same gene region.

Concluding Remarks—We can conclude that the small subunit of the amine dehydrogenase from both Ps. putida strains is a strongly acidic polypeptide (11 acidic versus 3 basic residues) with a highly basic C-terminal end, but of which the most peculiar characteristic is the occurrence of total four internal cross-linkages. Two of these occur between a cysteine residue and the side chain of a glutamic acid residue, one of which occurs with a glutamic acid residue, and one occurs between a cysteine residue and a carbon atom of the aromatic six ring of a tryptophan residue, which itself occurs in the oxidized quinone form. The x-ray data (in preparation) reveal that it is the γ methylene group of the glutamic acid and a carbon atom of the tryptophan that are involved in the thioether bond. To our knowledge, the covalent bond formation between a cysteine S-atom and a methylene group of an acidic residue or of a (modified) tryptophan has not yet been documented before, although such a bond with a histidine side chain has been identified in tyrosinase (20) and in gastropodan hemocyanin (21). Because of the strong nature of these types of bonds it seems to be excluded that they can be formed in vitro or in vivo without the presence of specific enzymes, the activity of which will have to be demonstrated. The complete tertiary structure of the amine dehydrogenase will very much allow understanding the structural implications of the intrachain cross-linkages and unraveling the catalytic mechanism of the enzyme.

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