Molecular characterization of male and female rat liver aldehyde oxidase is reported. As described for the mouse liver, male and female rat liver expressed kinetically distinct forms of aldehyde oxidase. Our data suggest that the two forms arise as a result of differences in redox state and are most simply explained by expression of a single gene encoding aldehyde oxidase in rats. In support of this argument we have sequenced cDNAs from male and female rat liver. We examined mRNA expression by Northern blot analysis with RNA from males and females, from several tissues, and following androgen induction. Purified rat liver enzyme from males or females revealed a single 150-kDa species consistent with cDNA sequence analysis. Both male and female forms were reactive to the same carboxyl-terminal directed antisera. K_{m(app)} values obtained in crude extracts of male or female rat liver and post-benzamidine-purified aldehyde oxidase differed substantially from each other but could be interconverted by chemical reduction with dithiothreitol or oxidation with 4,4'-dithiodipyridine. Our data indicate that a single gene is most likely expressed in male or female rat liver and that the kinetic differences between male and female rat liver aldehyde oxidases are sensitive to redox manipulation.

Aldehyde oxidase (AOX) is a native of the molybdenum cofactor containing enzymes. Native AOX is routinely prepared as a homodimer of 300 kDa. Each 150-kDa subunit contains two iron-sulfur centers, an FAD, and the molybdenum-pterin cofactor (MoCo) (1–3). AOX (EC 1.2.3.1) catalyzes the oxidation of a wide range of aldehydic compounds, purines, quinoliniums, and numerous pharmacologic agents. While substrate specificity for AOX is very broad, and wide species variation in substrate specificity exists, the general catalytic reaction takes the form of hydroxyl transfer from water to an aldehyde creating the cognate acid. For example, conversion of benzaldehyde to benzoic acid is a very efficient reaction for AOX from most species. Conversion of N-1-methyl nicotinamide (NMN) to the 2- or -4-pyridone has been used as a standard definition of AOXs, although it is usually a kinetically less efficient reaction than benzaldehyde oxidation.

AOX is of interest both for its role in drug metabolism and as a source of the reactive oxygen species (ROS), hydrogen peroxide, and the superoxide anion, that have been related to numerous human pathologies. The human gene encoding AOX has been linked to a rare form of amyotrophic lateral sclerosis, although it is unknown if AOX encodes the amyotrophic lateral sclerosis locus itself (4–6). ROS are generated from AOX in an oxidative half-reaction following reduction of the enzyme by substrate. The FeS, FAD, and MoCo cofactors comprise an internal electron transfer chain in which electrons are passed from the active site molybdenum center to the FeS centers and finally to FAD. Partial reduction of oxygen at the the reduced FeS site produces ROS. Unlike the related enzyme xanthine dehydrogenase (XDH), AOX does not utilize NAD+ as a cofactor and therefore AOX does not undergo the classical “D-form” to “O-form” conversion characteristic of XDH (7).

Two distinct AOX activities have been reported for mouse liver (8). Hepatic AOX from male and female mice differed in $K_m$ and $V_{max}$ for use of the substrate, benzaldehyde. The male enzyme exhibited a $K_m$ of 40 $\mu$M while the female enzyme had a $K_m$ of 115.4 $\mu$M. The male $V_{max}$ was 423.7 nmol/min/mg of protein and the female enzyme was 203.0 nmol/min/mg of protein. Furthermore, male and female mouse AOX enzymes were dramatically regulated by testosterone. Treatment of female mice with testosterone propionate (TP) converted both $K_m$ and $V_{max}$ values to the male pattern (8). Previous work had shown that castration resulted in the loss of a male pattern with conversion to a female pattern and TP supplementation restored the male-specific pattern of AOX expression (9). This observation was consistent with early reports of androgenic regulation of mouse hepatic AOX (10, 11). More recently, regulation of mouse hepatic AOX by testosterone was found to be mediated by growth hormone (12) where, again, growth hormone supplementation was found to convert $K_m$ and $V_{max}$ of the female-specific pattern to a male-specific pattern. Nonetheless, when male and female hepatic AOXs were purified to homogeneity they revealed a single band of 150 kDa on SDS-PAGE analysis and a single active band on native PAGE (8). How the differences between male and female hepatic AOXs were generated remained unclear.
Two distinct AOX activities were also purified from rat liver and identified as NMN oxidases I and II (13, 14). The two activities exhibited different \( K_m \) values for the oxidation of NMN to either its 2- or 4-pyridone. These two activities also differed by pH optima, heat stability, and inhibitor sensitivity. NMN oxidases I and II were found to possess distinct kinetic parameters, \( K_m \) and \( V_{max} \), for oxidation of several different substrates, including benzaldehyde and NMN. Furthermore, wide variation in substrates, including benzaldehyde and NMN. Furthermore, parameters, differed by pH optima, heat stability, and inhibitor sensitivity.

AOX genes have been identified in Arabidopsis thaliana (20), and to date, different AOX cDNA sequences were reported for corn plants that were themselves 83% identical (19). Three cDNA sequences were reported for corn plants. Two distinctly different forms of AOX in male and female rat livers. We have confirmed the existence of two kinetically different forms of AOX in male and female rats. Sequence analysis of the corresponding cDNAs indicates that a single AOX gene is most likely activated in the liver in male and female rats. By Northern blot analysis, male and female rat liver RNA contained a single mRNA species that did not exhibit induction by TP. Purified rat liver AOX from males and females revealed a single 150-kDa band on SDS-PAGE. Present experiments suggest that a primary difference between male and female forms of AOX may lie in their respective redox states.

**MATERIALS AND METHODS**

**RNA Purification and cDNA Synthesis**—RNA was prepared from organs of freshly killed Sprague-Dawley rats by quick freezing the tissue in liquid nitrogen followed by extraction in guanidine isothiocyanate and phenol: chloroform:isoamyl alcohol (24:24:1) (29). Frozen tissues were stored at –70 °C until use. Poly(A)+ RNA was prepared by fractionation on oligo(dT)-cellulose (Stratagene, La Jolla, CA). cDNA was prepared by reverse transcription in a final volume of 20 \( \mu \)l as follows. 1.0 \( \mu \)l of poly(A)+ RNA was mixed with diethyl pyrocarbonate-treated water, 1.0 \( \mu \)l of primer oligonucleotide at 20 \( \mu \)M, 1.0 \( \mu \)l of 10 mm deoxyribonucleoside triphosphates (ACGT), 0.5 \( \mu \)l of RNase inhibitor, 1.0 \( \mu \)l of recombinant Moloney murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Palo Alto, CA), and 4.0 \( \mu \)l of 5 \( \times \) buffer (final conditions: 50 m M Tris-HCl, pH 8.3, 75 m M KCl, 3 mm MgCl\(_2\)). Reactions were incubated at 42 °C for 60 min and then heated to 94 °C for 5 min to inactivate reverse transcriptase. Prior to use, reactions were diluted to 100 \( \mu \)l and 5 \( \mu \)l was used for PCR amplification. Reverse transcriptase reactions were stored at –70 °C.

**3’ and 5’ RACE**—A region from the middle of AOX1 was obtained by PCR amplification of reverse transcribed male rat liver poly(A)+ RNA using synthetic oligonucleotides (Life Technologies, Inc., Gaitherburg, MD) derived from a fragment of the mouse liver AOX1 sequence (27). Nucleotides 1,682 through 2,217 were amplified with the oligonucleotides MAO4RAT1 and MAO4RAT2 (Table I) to produce a single 535-bp fragment that was gel purified, sequenced in its entirety from both directions, and cloned as pMID. The resulting sequence was used to derive the unique sequence oligonucleotides for rat AOX1, FORINRAO, and REVINRAO. 3’ RACE was performed as follows. Male rat liver poly(A)+ RNA was reverse transcribed using the oligonucleotide 3’ RACE primer as a reverse primer for reverse transcriptase. The resulting single strand DNA was amplified by PCR using the oligonucleotides MAO4RAT1 and 3’ RACE. A second round of PCR amplification was performed using the 5’ nested oligonucleotide, REVINRAO, and 3’ RACE. A single product of 2,180 nt was obtained and sequenced entirely from both directions. This fragment was cloned as p3’ RACE and showed 100% identity with the overlap region of pMID.

**Table I**

| Strain | Oligonucleotides used for amplification of male and female rat AOX1 |
|--------|---------------------------------------------------------------|
| 3’-RATRACE | 5’-CCCGGGAGAATTCCCTGGACGTCGGT (T30) VN-3’ |
| 3’-RATUTR | 5’-CCCGGGGAAATCTCGGGAGGCTTGCTTGTGTTGGACCAAATCC-3’ |
| MAO4RAT1 | 5’-CTGGAGTACATTAAAGATACCAAGATG-3’ |
| MAO4RAT2 | 5’-GTATTTTCACTCTCAAGATTGATC-3’ |
| FORINRAO | 5’-GTCGATCCAGAGGCTTGGTATGG-3’ |
| REVINRAO | 5’-GCCTGGGACGACGCTTTGGTATG-3’ |
| 3’-IVS10 | 5’-CATCCAAAACGGCGTGTGGCCGAGTC-3’ |
| 3’-IVS11 | 5’-CCGAGCTTGGGAGGACTCTGATG-3’ |
| IVS22 | 5’-CCCGGCGTTCCTTCTTACGTTAAGCGG-3’ |
| RAT5 | 5’-ATCTCCTTCTCGTAAATTCGTCCGGATCC-3’ |
| RAT6 | 5’-CCCGGTACGGCGGGTCCTTCTTCGACG-3’ |
| 3’-REND | 5’-CCCGGGGAATTCCTGCAGGTCGACGCGTTCTGTAGTTGTTGAGCCAATCC-3’ |

**Note:** Oligonucleotides were used for amplification of male and female rat AOX1.
adaptor-ligated single strand DNA. Adapter-ligated single strand-DNA was subjected to first round amplification using the oligonucleotides 3’ IVS11 and RAT5. The resulting PCR products were subjected to a second round of amplification using 3’ IVS11 and the nested primer, RAT6. The resulting 160-base pair DNA was cloned (p5’ RACE-male), sequenced, and showed 100% identity with the overlap region of p5’ RACE. This sequence was inferred to contain the translation initiation site and 49 nucleotides of 5’-untranslated region because it showed excellent deduced amino acid sequence homology with human and bovine AOX sequences, a single ATG was found to be in-frame with the downstream sequence, and translational termination sequences were observed upstream of this ATG and in the same reading frame.

DNA Sequence Analysis—Direct fluorescence sequence analysis was performed on plasmid DNA or PCR products using oligonucleotide primers designed to yield approximately 400 nucleotides between oligonucleotide primers. Each fragment was sequenced entirely from both directions. A list of sequence analysis primers and their sequences is available upon request. The assembled cDNA sequences for both male and female rat liver AOX have been deposited in the NCBI gene bank data base.

adapter-ligated single strand DNA. Adapter-ligated single strand-DNA was subjected to first round amplification using the oligonucleotides 3’ IVS11 and RAT5. The resulting PCR products were subjected to a second round of amplification using 3’ IVS11 and the nested primer, RAT6. The resulting 160-base pair DNA was cloned (p5’ RACE-male), sequenced, and showed 100% identity with the overlap region of p5’ RACE. This sequence was inferred to contain the translation initiation site and 49 nucleotides of 5’-untranslated region because it showed excellent deduced amino acid sequence homology with human and bovine AOX sequences, a single ATG was found to be in-frame with the downstream sequence, and translational termination sequences were observed upstream of this ATG and in the same reading frame.

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AOX-CT (carboxyl-terminal antibody).

Western Immunoblot Analysis—Protein was electrophoresed on SDS-PAGE and transferred to polyvinylidine difluoride membranes (Bio-Rad). Filters were sliced for staining with Commassie Brilliant Blue or processed for immunoblot analysis. For reaction with antisera, filter strips were blocked with gelatin overnight prior to reaction with pre-immune sera, AOX-NT, or AOX-CT antisera. Antigen-antibody complexes were detected by reaction with alkaline phosphatase streptavidin kit (Bio-Rad).

RESULTS

Different Forms of AOX Exist in Male and Female Rat Livers—AOX enzyme activity was measured in crude extracts of rat liver.

Clustal analysis. Identical amino acids are boxed in black and biochemically conserved differences are shown in gray. Regions thought to mediate cofactor binding are indicated by the overline. Five sites within the large MoCo-binding domain have been identified and are shown individually. The 5 amino acid differences between male and female rat liver AOXs have been indicated with an asterisk. The programs ClustalW, Boxshade, and Paint were used to create the figure.

FIG. 2—continued

Amino acid sequences for the four vertebrate AOXs have been aligned by multiple Clustal analysis. Identical amino acids are boxed in black and biochemically conserved differences are shown in gray. Regions thought to mediate cofactor binding are indicated by the overline. Five sites within the large MoCo-binding domain have been identified and are shown individually. The 5 amino acid differences between male and female rat liver AOXs have been indicated with an asterisk. The programs ClustalW, Boxshade, and Paint were used to create the figure.
male and female rat livers. Apparent $K_m$ ($K_{\text{app}}$) values were determined from Lineweaver-Burk plots by measuring conversion of NMN to its pyridone. $K_{\text{app}}$ for male rat liver AOX was 538.8 $\mu$M and $K_{\text{app}}$ for the female was 1062.3 $\mu$M, consistent with previous reports showing two forms of AOX in livers from rats and different forms of AOX in livers from male and female mice. While no explanation for this difference has been produced, the two AOX genes identified in mice, AOX1 and AOX2, suggested the possibility that two different AOX genes may be expressed in rat liver.

**cDNA Sequence Analysis of Male and Female Rat Liver AOX1**—Fig. 1 illustrates the PCR amplification strategy used to obtain segments of male rat liver AOX1. DNA sequence of the three PCR products was assembled to produce the male rAOX1 cDNA. RAOX1 comprised 4,304 nucleotides, including 30 nt of polyadenylation, 210 nt of 3'-untranslated region, and 49 nt of 5'-untranslated region. A single open reading frame was identified that encoded a protein of 1,333 amino acids and a deduced mass of 147,009 Da. The deduced male rAOX1 protein exhibited 82% sequence identity with human AOX1 and 81% sequence identity with bovine AOX1. Multiple sequence clustal analysis revealed conservation of co-factor domains corresponding to FeS I, FeS II, FAD, and five small domains within the MoCo binding segment (Fig. 2).

**TABLE II**

Sequence differences in male and female rat AOX1 clones described here

| No. | nt-Site | Base pair change | Amino acid change | Amino acid site |
|-----|---------|------------------|-------------------|----------------|
| 1   | 133     | G:C–A:T          | P–P               | 119            |
| 2   | 408     | C:G–G:C          | A–G               | 120            |
| 3   | 408     | G:C–T:A          | R–M               | 649            |
| 4   | 1,679   | T:A–C:G          | L–L               | 1276           |
| 5   | 1,994   | A:T–G:C          | T–A               | 1,276          |
| 6   | 2,563   | G:C–A:T          | L–L               | 1,315          |
| 7   | 2,872   | T:A–C:G          | S–S               | 1,276          |
| 8   | 3,739   | G:C–A:T          | Q–Q               | 1,276          |
| 9   | 3,875   | C:G–T:A          | L–F               | 1,276          |
| 10  | 3,993   | G:C–C:G          | R–T               | 1,276          |

Female rat liver AOX1 cDNA sequence was obtained using a similar strategy with the exception that a unique sequence oligonucleotide derived from the male sequence, 3' RATUTR, was used to obtain the 3' RACE product. Thus, 47 nucleotides of 3'-untranslated region was obtained for the female and this does not include the polyadenylation site (Fig. 1). The assembled cDNA sequence for female rat liver AOX1 encoded a deduced protein of 1,333 amino acids and 146,919 Da. The female cDNA sequence was 99.8% identical to the male sequence and the deduced protein sequence was 99.6% identical to the male sequence. Of the 10 nucleotide differences detected between the male and female rat liver AOX1 cDNA sequences described here, five resulted in changes to the deduced amino acid sequences (Table II). However, while nucleotides 405 and 408 differ between male and female sequences reported here and to the GenBank data base, these variations were also found between individual male clones and therefore do not represent gender differences but differences between individual rats. The full extent of individual variation was not determined and it remains possible that all of the differences observed between the two clones described may be attributed this cause alone.

**Expression of Rat AOX1 mRNA**—Fig. 3A shows Northern blot analysis of poly(A)$^+$ RNA from male rat liver. Hybridization probes were derived from each of the three male clones, pMID, p5'RATRACE, and p3'RATRACE. The region between nucleotides 1,682 and 2,217 of the male cDNA, corresponding to the pMID hybridization probe, produced hybridization signals at approximately 4,500 and 2,500 nt. Hybridization probes derived from both the p5'RATRACE and p3'RATRACE clones produced predominantly a single band at 4,500 nt with weak hybridization to the band at 2,500 nt. We conclude that the predominant mRNA for AOX1 detected by Northern blot analysis in males is approximately 4,500 nt, consistent with the cDNA sequence assembled for rAOX1. The unexpected signal at 2,500 nt may represent a cross-reactive species largely localized to the pMID region. Hybridization probes derived from the p5'RATRACE produced predominantly a single band from both male and female RNA (Fig. 3B). This RNA was also estimated to be 4,500 nucleotides in size, and no difference in size or number of hybridizing bands was detected between males and females.

Northern blot analysis of poly(A)$^+$ RNA from several different tissues showed expression of a single 4,500-nt RNA for all tissues examined (Fig. 3C). Different tissues did not show variation in either the size or multiplicity of AOX RNAs. Var-

![Fig. 3. Northern blot analysis of rat AOX1 expression.](image-url)
TABLE III
Quantitation of RNA hybridization

Hybridizing bands from the Northern blot shown in Fig. 4 were cut from the filters and counted by liquid scintillation counting. Counts from randomly selected regions were averaged and subtracted from each signal to account for background radioactivity. Counts for each AOX signal were then normalized to the β-actin signal derived from the same lane, and the normalized, background subtracted counts were multiplied by 100. The number of animals in each group is shown by the n. The mean and standard error of the mean (SE) were calculated for each group. No statistical significance could be established between group means.

| Group                 | n | Mean | S.E. |
|-----------------------|---|------|------|
| Sham castrated        | 4 | 69.0 | 10.3 |
| Castrated control     | 4 | 86.6 | 11.4 |
| Castrated testosterone| 5 | 82.9 | 18.3 |
| Castrated growth hormone| 5 | 64.9 | 13.5 |

Fig. 4. Testosterone does not regulate steady state levels of rat hepatic AOX. Poly(A)⁺ RNA from individual male rat livers has been analyzed by Northern blot. Individuals from the following four groups have been used: castrated, sham castrated, castrated and testosterone supplemented, castrated and growth hormone supplemented. β-Actin was used as a control to reveal uniform loading of RNA. Blots were first probed with the AOX probe, the corresponding region cut from the filter for counting, and the remaining filter was dissociated of all 32P and rehybridized with the β-actin probe.

Dissociation in the β-actin control precludes drawing firm conclusions at this point concerning relative levels of expression between tissues.

RNA from male rats that had been sham castrated, castrated, castrated and treated with TP, or castrated and treated with growth hormone was analyzed by Northern blot. Fig. 4 shows that steady state RNA levels were only slightly affected by any of these treatments. When hybridization signals were quantitated and normalized to either OD 280 or to β-actin hybridization signal (Table III), we found no statistically significant difference between groups in AOX1 mRNA abundance. These data do not support significant regulation of rat AOX1 mRNA abundance by TP.

AOX Enzyme Purification and Characterization—AOX enzyme activity was purified to homogeneity from male rat livers (Fig. 5A). These preparations produced a single band of 150 kDa by SDS-PAGE analysis. We were unable to sequence this protein by direct Edman degradation suggesting that its amino terminus was blocked, as was found for both the rabbit liver and bovine liver AOXs (5, 26). Western immunoblot analysis of partially purified AOX from male and female rat livers revealed excellent reactivity of each enzyme preparation to this synthetic peptide-derived antibody (Fig. 5B). Furthermore, reactive proteins from male and female revealed a predominant polypeptide of approximately 150 kDa with no evident difference in size between genders. (Fig. 5C).

K_m(app) values for oxidation of NMN to its pyridone were determined by analysis of Lineweaver-Burk plots. Fig. 6 and Table IV show these results. As noted above, crude liver extracts from males produced K_m(app) of 538.8 and 1062.3 μM for females. Reduction of the crude liver extract with 5 mM DTT shifted K_m(app) for males and females. Reduced male rat liver produced K_m(app) of 359.9 μM and for the reduced female enzyme K_m(app) was 354.5 μM (Fig. 6B). Thus, male and female forms of AOX can be converted to a form with indistinguishable K_m(app) values by chemical reduction in a crude lysate. Oxidation of the reduced female preparation with 4,4'-DTDP converted K_m(app) back to a form similar to that obtained from the untreated female preparations (Fig. 6B). Furthermore, AOX purified from female rat liver through the post-benzamidine stage could be reduced with DTT to yield an enzyme with K_m(app) of 261 μM. Reoxidation of the reduced enzyme with 4,4'-DTDP converted the K_m(app) to 1673 μM (Fig. 6C). Thus, the capacity to modulate K_m(app) of rat liver AOX was maintained through purification of the enzyme and may therefore reflect an intrinsic property of the enzyme.

DISCUSSION

In the present work have assembled full-length sequences for AOX cDNAs from male and female rat liver. We examined expression of AOX by Northern blot analysis from males and females, from several tissues, and have examined the effect of androgen regulation. We purified AOX from rat livers of males and females and obtained a single 150-kDa band by SDS-PAGE analysis consistent with the deduced amino acid sequences from males or females of 1,333 amino acids and 147 kDa. Antibodies raised against synthetic decapeptides reacted with AOX from males or females. Furthermore, K_m(app) values for crude extracts of male or female rat liver and post-benzamidine purified AOX differed substantially but could be interconverted by chemical reduction with DTT or oxidation with 4,4'-DTDP.

The deduced amino acid sequences for male and female rat liver AOX are 81 and 82% identical to human and bovine AOXs and are themselves 99.6% identical. They show excellent conservation in the domains attributed to iron, FAD, and MoCo binding. By this criterion, the rat AOXs clearly belong to the molybdenum iron-sulfur flavoproteins that include AOX, XDH, and XO. Furthermore, the rat AOXs conform to the domain models proposed for XDHs (7, 30, 31). Amino acids critical for catalysis of this class of enzymes are conserved in the rat AOX sequences as they are in most other AOXs and XDHs. In particular, Glu-869 of the Mop enzyme is critical for catalysis and is conserved in nearly all XDHs and AOXs (see Ref. 21 for alignment of several sequences), including the two rat sequences reported here where it is found at amino acid 1265 in MoCo domain 5 (Fig. 2).

We observed that the male and female cDNA sequences were not identical. While several arguments could be advanced to explain the differences, we suggest that the least tenable ar-
The amino-terminal sequence analysis revealed that purified AOX was analyzed by SDS-PAGE and stained with Ponceau S prior to subjecting the enzyme to aminoterminal sequence analysis. Antibody was raised against synthetic decapeptides from the amino (AOX-NT) and carboxy (AOX-CT) termini. AOX trimming was partially purified to retain numerous unrelated proteins, analyzed by SDS-PAGE (Crude AOX), and reacted to each antibody as described under “Materials and Methods.” Preimmune, AOX-NT, and AOX-CT antisera were used at a 1:500 dilution. Male and female rat liver AOX was purifi ed to homogeneity from initially reduced preparations. Enzymes were analyzed by SDS-PAGE and subjected to Western immunoblot analysis using the AOX-CT antisera. Both male and female preparations migrated with apparent size of 150 kDa and both preparations reacted to the AOX-CT antisera.

Furthermore, sequence polymorphism is a well described phenomenon in Drosophila XDH (32–34). Multiplicity of AOX genes has been established for some organisms (19–24), and in plants these data are supported by multiple cDNA sequences of approximately 80% identity (19, 20). Thus, the observation that mice appear to express two AOX genes in the liver was not surprising (9). However, genetic data derived in the mouse have not been supported yet by corresponding molecular data. Although efforts have been made to establish AOX gene copy number in vertebrates, Southern blot analysis of chromosomal DNA failed to reveal second AOX genes in several vertebrates under conditions in which at least 80% identity would have produced hybridization (28).

Northern blot analysis of AOX mRNA from male or female rats, and from several different tissues, demonstrated expression of a single 4,500 nt RNA. We did not observe variation in size or multiplicity of AOX mRNAs in the liver from males or females where kinetically distinct forms of AOX were identified. Furthermore, castration and/or testosterone supplementation resulted in no significant alteration in AOX mRNA abundance and we infer that testosterone does not appear to exert significant regulation of AOX mRNA abundance or form in the rat liver. We did observe surprisingly strong hybridization to an RNA of 2,500 nt that could be localized to the region from +1,682 to +2,217. While the identity of this RNA is unknown, it is too small to encode an AOX of 150 kDa. Thus, RNA analysis supports expression of a single AOX gene in the liver where post-translational events may be important for determining the differences between male and female kinetic variants.

Interestingly, AOX-3 from A. thaliana encoded a protein of only 568 amino acids truncated at the amino terminus (20). It must differ from a true AOX because it cannot encode a protein capable of binding the full set of co-factors. AOX-3 from A. thaliana showed striking homology to AOX-1 and AOX-2 in the MoCo-binding region, confirming that it is indeed a member of the MH family. Confirmation that vertebrates encode such a protein would be of great interest since it may represent the RNA detected at 2,500 nt.

Since our observations suggested that rats express a single AOX gene in the liver, we examined the possibility that redox status might underlie the differences in male and female variants. We found that partially purified crude extracts of male and female rat liver did indeed reveal different K_{m(app)} variants of AOX. Reduction of crude extracts from males or females with DTT resulted in conversion to a single form. Subsequent reoxidation of the reduced AOX with 4,4'-DTDP resulted in conversion to a more female like K_{m(app)}. Reduction or oxidation of post-benzamidine purified AOX also resulted in interconversion between the two extremes of K_{m(app)}, suggesting that the variants differed by their intrinsic oxidation state. Since DTT directly reduces protein disulfides to thiols while 4,4'-DTDP forms disulfides from thiols (35–38), we infer that manipulation of thiol oxidation state can interconvert kinetic variants of AOX.

Redox effects on XDH are well known. Conversion between the NAD+ dependent, D-form, and the oxygen dependent, O-form, is a redox-dependent process reversible by chemical reduction (7, 39, 40). Furthermore, redox effects on XDH have dramatic effects on the kinetic parameters of the enzyme (41). AOX does not have an NAD+-dependent form of the enzyme, and therefore conversion between D-form and O-form is irrelevant. While cysteine residues critical for D-form to O-form conversion in XDH were not conserved in AOX (42), most of the 41 cysteine residues found in rat liver AOX are conserved with vertebrate XDHs. These cysteine residues would be expected to take part in the same biochemical reactivities as those found in XDH. Since we observed that redox effects on K_{m(app)} were preserved from crude extracts through post-benzamidine-puri-
Chemical reduction or oxidation abolishes differences in $K_{m(app)}$ between male and female rat liver AOX. AOX activity was purified from unreduced male and female rat livers through (NH$_4$)$_2$SO$_4$ fractionation and either treated with 5 mM DTT or not to produce the crude native or crude reduced preparations. A separate preparation was reduced initially and purified through benzamidine fractionation to produce the post-benzamidine preparation. A, Lineweaver-Burk plots were determined for male (squares) and female (diamonds) preparations using NMN as the oxidizing substrate. B, Lineweaver-Burk plots were determined for initially reduced male and female crude extracts. In addition, the reduced female extract was then treated with 1 mM 4,4′-DTDP to reoxidize it (open squares) and subjected to Lineweaver-Burk analysis. C, female rat liver AOX was purified through benzamidine fractionation from an initially reduced extract. Enzyme was treated with 5 mM DTT or 1 mM 4,4′-DTDP to reduce or oxidize the enzyme. Lineweaver-Burk analysis was then conducted on the reduced (diamonds) or oxidized (squares) preparations.

Our observations can be explained by the activity of the hepatic microsomal monooxygenase. The cystamylating flavin monooxygenase catalyzes oxidation of cysteamine, a thiol, to cystamine, a disulfide, and this reaction provides a significant source of disulfide responsible for maintaining the intracellular thiol:disulfide potential (43, 44). The thiol:disulfide potential is thought to reflect two ratios: the GSH:GSSG ratio and the cysteamine:cystamine ratio. Protein oxidation state depends on overall thiol:disulfide potential. Higher levels of monooxygenase lead to a more oxidizing environment, while reduced monooxygenase levels result in a more reducing environment. Significantly, monooxygenase from mice or rats is regulated by testosterone in precisely the fashion that kinetic variants of AOX are regulated (43). Male rat hepatic monooxygenase levels are lower than female levels. Castration of male rats elevates monooxygenase and leads to elevated cystamine and a more oxidizing cytosol. Testosterone reverses this effect and restores the reducing environment. Furthermore, testosterone treatment of females lowers monooxygenase and elevates cysteamine levels. These observations may explain both the effect of testosterone and the ability to purify kinetically distinct forms of AOX from male and female rats since neither thioltransferase nor glutathione reductase, the two other enzymes establishing thiol:disulfide potential, are regulated by testosterone (43). We posit that AOX is sensitive to the thiol:disulfide potential and that kinetic variants of AOX reflect the intracellular thiol:disulfide potential through thiol modification of AOX. Activity of the monooxygenase could thereby regulate AOX $K_{m(app)}$ by “setting” the thiol:disulfide potential leading to a more oxidized form of AOX in the female or a more reduced form of AOX in the male. This may have direct consequences for ROS generation from AOX since the kinetically less efficient enzyme may bias ROS generation for superoxide anion. This argument does not require expression of a second AOX gene and can account for the failure of testosterone to regulate AOX gene expression in rats despite finding kinetically distinct forms in males and females. AOX thiol modification could also explain both novel electrophoretic variants (9) and the widely variant kinetic characteristics of AOX from different organs and different species (16, 45–47).

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