Biochemical and Biophysical Research Communications **282,** 297–305 (2001)
doi:10.1006/bbrc.2001.4537, available online at http://www.idealibrary.com on IDEAL®

Microelectrospray Ionization Analysis of Noncovalent Interactions within the Electron Transferring Flavoprotein

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Received February 12, 2001

Cofactor associations within the electron transferring flavoprotein (ETF) were studied in real time using microelectrospray ionization-mass spectrometry (μESI-MS). Initial analysis of porcine (pETF) and human ETF (hETF) revealed only the holoprotein. When μESI-MS source energies were increased, both pETF and hETF readily lost AMP. Analysis of hETF and pETF in methanol revealed intact α- and β-subunits, and β-subunit with AMP. The pETF also contained β-subunit with FAD and β-subunit with both cofactors. In contrast to crystal structure predictions, AMP dissociates more readily than FAD, and the pETF β-subunit has an intimate association with FAD. This work demonstrates the complementarity of μESI-MS with NMR X-ray and optical spectroscopy in the analysis of noncovalent complexes. © 2001 Academic Press

Key Words: **electron transferring flavoprotein; microelectrospray ionization; mass spectrometry; noncovalent interactions.**

Electron transferring flavoprotein (ETF) is an electron-acceptor protein located in the mitochondrial matrix (1). ETF interacts with and accepts electrons from at least ten different flavoprotein dehydrogenases to provide a link to the electron transport chain via ETF:ubiquinone oxidoreductase (ETF:QO) (2). Defects in human ETF (hETF) or ETF:QO cause glutaric acidemia type II, an often fatal disease characterized by deficiencies in fatty and amino acid metabolism (3). Mammalian ETF is reported to be a heterodimer consisting of an α-subunit and β-subunit with calculated molecular weights of 33,093 Da and 27,841 Da respectively (GenBank Accession Nos. J04058 and NM_001985) (4, 5). These subunits are nuclear encoded, synthesized in the cytosol, and imported into the mitochondria. Unlike the β-subunit, the α-subunit is synthesized as a precursor, and processed to the mature form in the mitochondria (6). In addition, the ETF holoprotein consists of one mole of flavin adenine dinucleotide (FAD) and one mole of adenosine monophosphate (AMP) per mole of heterodimer (7).

The crystal structure of hETF has been solved to 2.1 Å and consists of three domains (8). The amino-terminal portion of the α-subunit and the majority of the β-subunit comprise domains I and III respectively. Domain II contains the carboxy-terminal regions of the subunits. The FAD moiety lies in a cleft in the α-subunit with the isoalloxazine ring in a crevice between domains II and III with the xylene portion of the FAD pointing toward the β-subunit. The FAD prosthetic group is bound almost exclusively by the α-subunit with limited van der Waals interaction with the β-subunit. Residues in the immediate vicinity of FAD are highly conserved in the sequence alignment of the known ETFs. Domain III contains the AMP-binding site with AMP being deeply buried within that domain. Porcine ETF (pETF) fluorescence-emission is 6-fold higher than hETF (9), suggesting differences in flavin/subunit interactions. The fluorescence properties of pETF are utilized to assay for deficiencies of mitochondrial flavoprotein dehydrogenases (10).

Microelectrospray ionization (μESI) is regarded as a soft ionization technique with ionization energies <1 eV above ground state energy (11). Hence it is possible to spray solutions of protein mixtures and detect intact specific, noncovalent complexes in the mass spectrometer. Indeed, μESI-MS has proved to be a highly sensitive technique for the study of such biomolecular interactions and has been used to investigate protein-protein, protein-DNA, and protein-ligand/drug interactions (12–18). In the present work, we demonstrate the usefulness of this approach to rapidly investigate the specific noncovalent interactions of the ETF (human and porcine) subunits with their respective cofactors.

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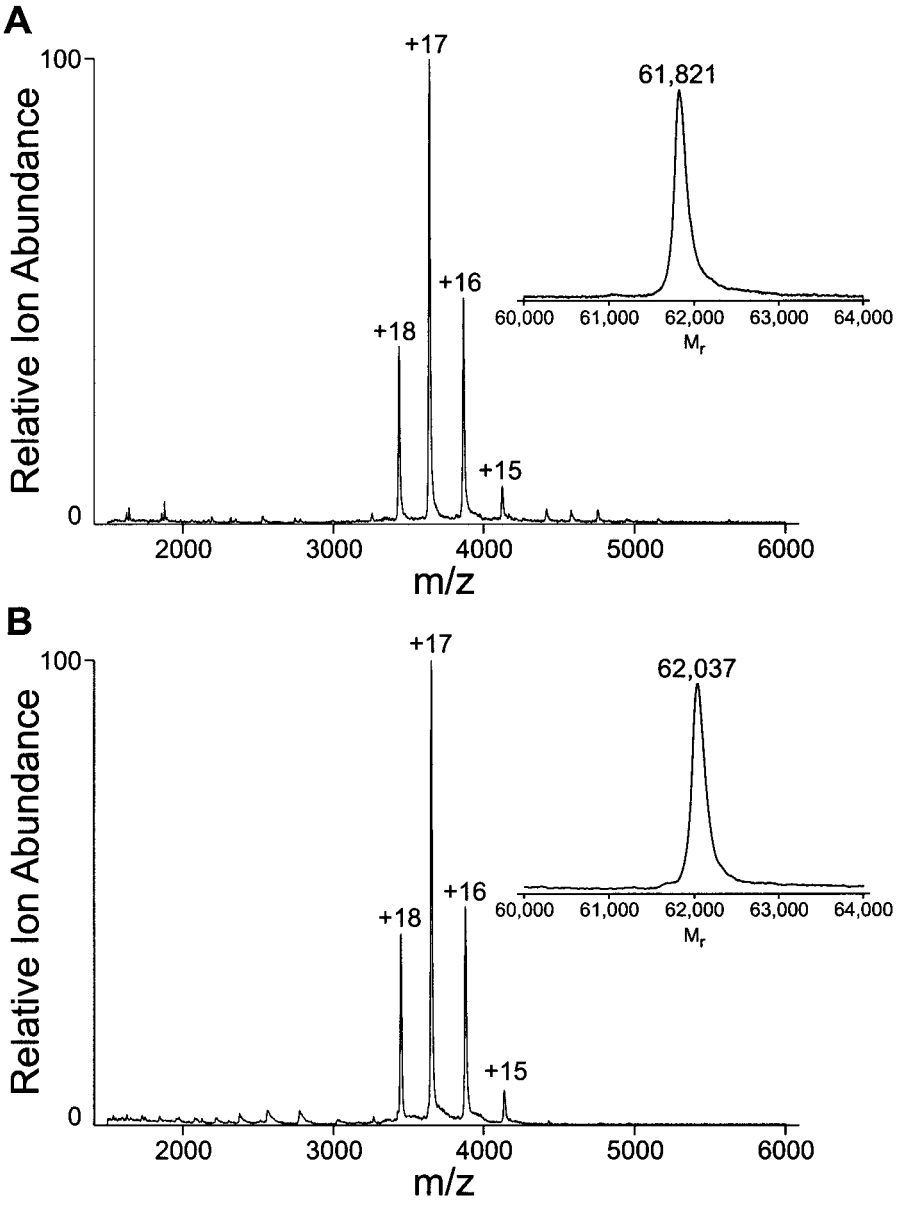

FIG. 1. Positive ion μESI-MS analyses of ETF holoproteins. (A) pETF and (B) hETF, both sprayed in 10 mM ammonium bicarbonate at pH 8.0. The charged states are numerically labeled. Insets show the relative molecular mass (M_r) of the transformed data.

MATERIALS AND METHODS

Protein purification. Porcine liver ETF was purified as previously described (19). The pBluscript SK+ plasmid containing the cDNAs encoding for the human ETF subunits was a generous gift from Dr. Frank Frerman, University of Colorado (9). The β-subunit sequence contains three nucleotide substitutions resulting in three amino acid changes (E3D, L4V, and D15G) in the amino-terminal portion of the subunit. The plasmid was transformed into *E. coli* host strain XL1-Blue (Strategene, Inc., La Jolla, CA). Cells were grown, harvested, and disrupted as previously described (20). Cell-free extracts were applied to an XK 50/20 DE52 column equilibrated with 10 mM potassium phosphate pH 7.5. The hETF eluted from the column in the same buffer. Yellow, fluorescing fractions were pooled and further purified on a 20 μM ceramic hydroxyapatite column equilibrated with 10 mM potassium phosphate pH 7.5. The hETF was eluted with a 350-mL gradient of 10–150 mM potassium phosphate pH 7.5. Fractions with an A_{270}/A_{436} ratio between 2 and 4 were pooled and concentrated to an A_{436} greater than 1. The hETF was stored in 20% glycerol at −20°C. Microcon 30 microconcentrators (Amicon, Inc., Bedford, MD) were used to buffer exchange the proteins into 10 mM ammonium bicarbonate pH 8 as described previously (14).

Microelectrospray ionization mass spectrometry. All mass spectra were obtained using a Finnigan MAT 900 mass spectrometer (Bremen, Germany) of EB geometry. A modified Finnigan MAT electrospray source was used as previously described (21). MicroESI measurements were obtained in positive ion mode with SF_6 introduced through the auxiliary gas port to prevent source corona discharge. The sample was introduced at 0.3 μl/min and sprayed from a fused silica emitter of 20 μm i.d. The μESI source voltage was at 3.2–3.7 kV, with a capillary temperature of 120°C or 200°C. The magnet (B)

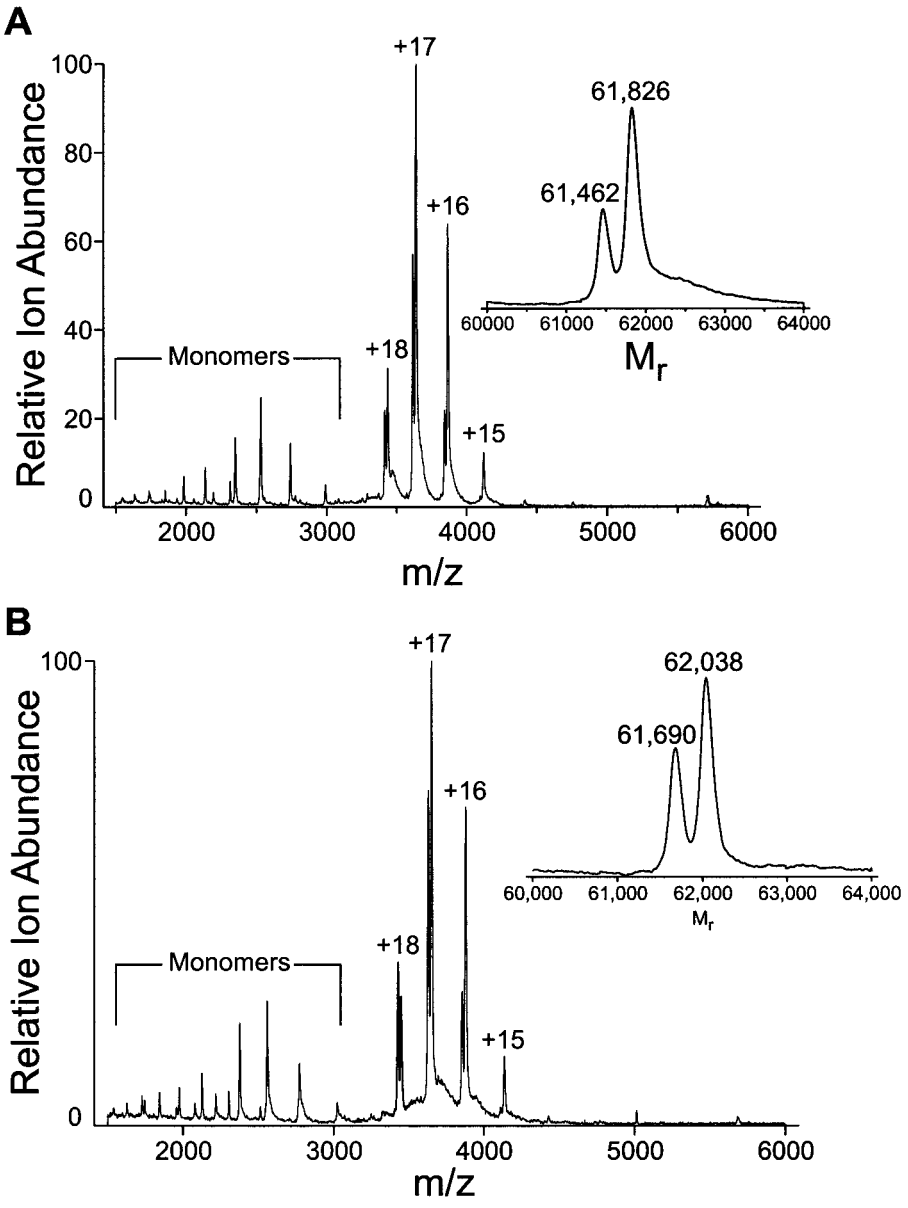

FIG. 2. Loss of AMP due to sCID of ETF holoprotein. Multiply charged positive ion μESI-MS spectra of (A) pETF and (B) hETF were obtained under sCID conditions. The charged states are numerically labeled. Insets show transformed spectrum.

was scanned from mass to charge (m/z) 1000 to 7000 at a rate of 5 or 10 s per decade. The position and time resolved ion counter (PATRIC) array detector with an 8% mass window was used for ion detection. Multiple scans were collected, summed, and the multiply charged spectra transformed to give relative molecular mass (M_r) values using the Finnigan MAT software.

RESULTS

Holoprotein Analysis

Initially both pETF and hETF holoproteins were subjected to μESI-MS under non-denaturing conditions. It should be noted that amino acid and DNA sequence for pETF have not, to date, been described. Analysis of pETF afforded a series of multiply charged ions (+15 to +18) between m/z ~3400–4500 as shown in Fig. 1A. Transformation of this ion series revealed a relative molecular mass (M_r) of 61,821 Da (±0.01%) (Fig. 1A, inset). The μESI-MS analysis of hETF holoprotein also yielded a similar series of multiply charged ions (+15 to +18) also between m/z ~3400–4500 (Fig. 1B). The transformed mass spectrum exhibited a M_r = 62,037 Da (±0.01%) (Fig. 1B, inset). In the case of hETF, the M_r value was different from the predicted value, based on the expression plasmid DNA sequence. However, amino-terminus Edman sequencing revealed that the expressed protein was missing

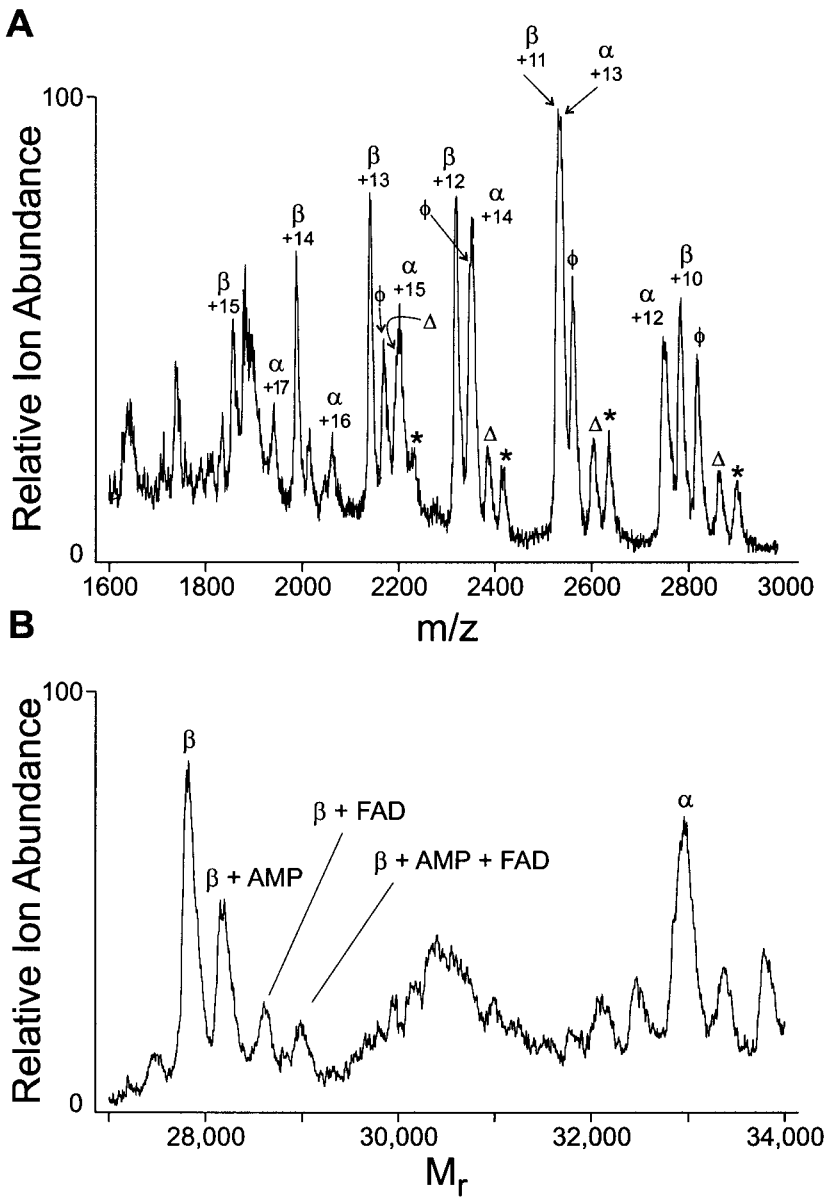

FIG. 3. Monomeric forms of porcine ETF in 20% methanol. (A) Multiply charged positive ion. (B) transformed μESI-MS spectra of porcine ETF. The sample was prepared in 10 mM NH_4HCO_3 pH 8.0 and 20% methanol. The charged states are numerically labeled; α-subunit (α); β-subunit (β); β-subunit with AMP (ϕ); β-subunit with FAD ($\triangle$); β-subunit with AMP and FAD (*). Unlabeled peaks in the transformed spectrum are artifacts of the reconstruction software package.

the initiator Met of the β-subunit. These changes brought the measured versus expected M_r within the experimental mass accuracy of $\pm$0.01%. It should be noted that in both the hETF and pETF, the M_r detected corresponds to the $[M + 3NH_3]^+$ ion (Figs. 1A and 1B) commonly seen in the presence of NH_4^+ salt spraying conditions.

Induced Holoprotein Dissociation

As noted above, pETF and hETF both contain one mole of FAD and one mole of AMP per mole of heterodimer (7). These cofactors are noncovalently bound to the holoprotein. To further elucidate the cofactor interactions, we increased the cap and tube voltages in the μESI source to induce skimmer collision induced dissociation (sCID) conditions (18). Under such conditions, two distinct ion series were observed for both pETF (Fig. 2A) and hETF (Fig. 2B) between m/z 3400 and 4500. On transformation of these multiply charged ions, in both cases, intact pETF (Fig. 2A, inset) and hETF (Fig. 2B, inset) holoprotein are still observed (M_r = 61,826 Da and 62,038 Da, respectively). However, for both pETF and hETF, another prominent M_r

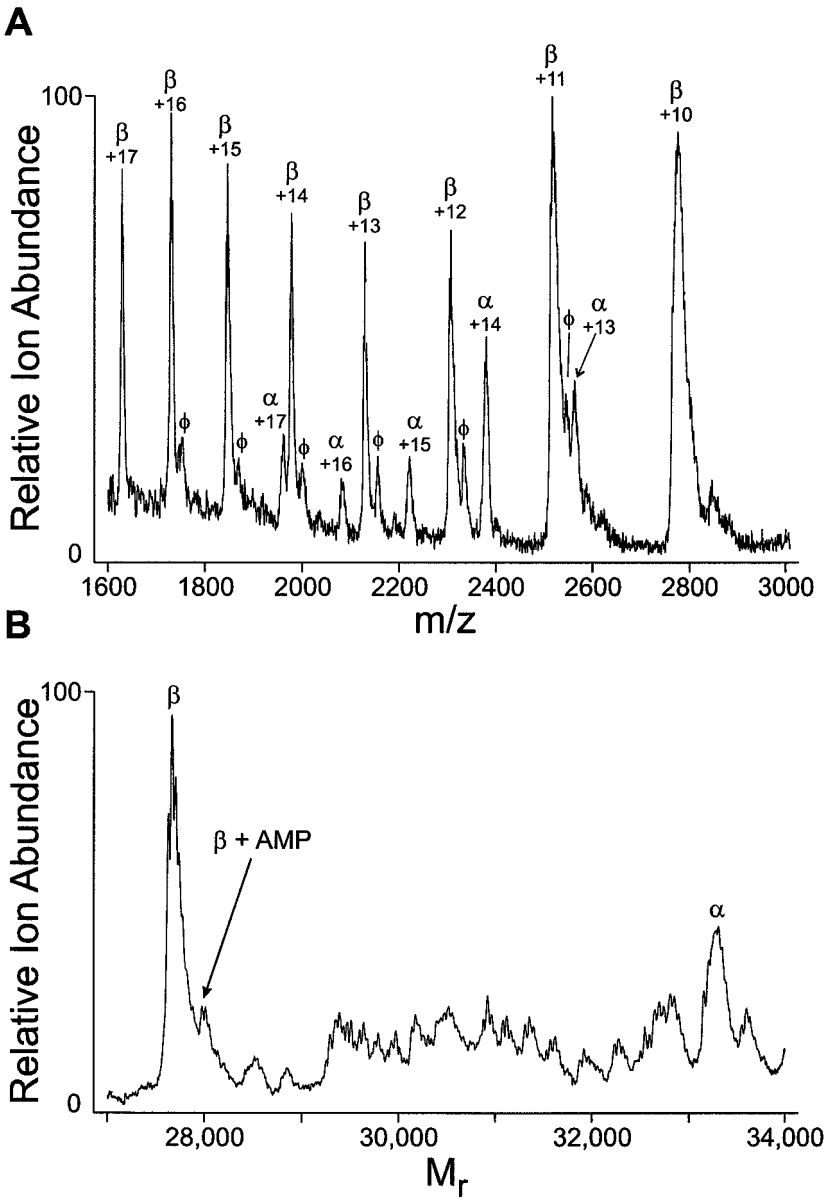

FIG. 4. Monomeric forms of human ETF in 25% methanol. (A) Multiply charged positive ion. (B) transformed μESI-MS spectra of human ETF. The sample was prepared in 10 mM NH_4HCO_3 pH 8.0 and 25% methanol. The charged states are numerically labeled; α-subunit (α), β-subunit (β), β-subunit with AMP (ϕ). Unlabeled peaks in the transformed spectrum are artifacts of the reconstruction software.

ion at 61,462 Da (loss of 364 Da) and 61,690 Da (loss of 348 Da), respectively, is also detected. When ETF is analyzed in the presence of high concentrations of ammonium bicarbonate, a mass difference of –364 Da is observed and corresponds to loss of AMP as its ammonium salt (Fig. 2A, inset). However when the protein is analyzed either in the presence of low concentrations of ammonium bicarbonate or 0.2% triethylamine (data not shown) a mass difference of only ~ -340 Da is observed, corresponding to loss of AMP alone (Fig. 2B, inset). However, if the protein is analyzed in the presence of 1 mM kCl, then AMP is lost as its K^+ salt (data not shown). For both pETF and hETF, AMP is readily lost either as AMP or as an AMP salt. Finally, it should be noted that the ion series between *m/z* 1000–3000 (Figs. 2A and 2B) represent the multiply charged ions of individual monomeric α and β subunits of the protein.

To further investigate the dissociation properties of the holoprotein, 20–25% methanol was added to the aqueous protein solutions. Analysis of pETF revealed a complex, multiply charged spectrum (Fig. 3A), which on transformation revealed cation adducted intact α-subunit (32,958 Da) and β-subunit (27,824 Da) as well as β-subunit with AMP (28,176 Da), β-subunit with FAD (28,605 Da), and β-subunit with both cofactors (28,969 Da) as shown in Fig. 3B. Analysis of hETF

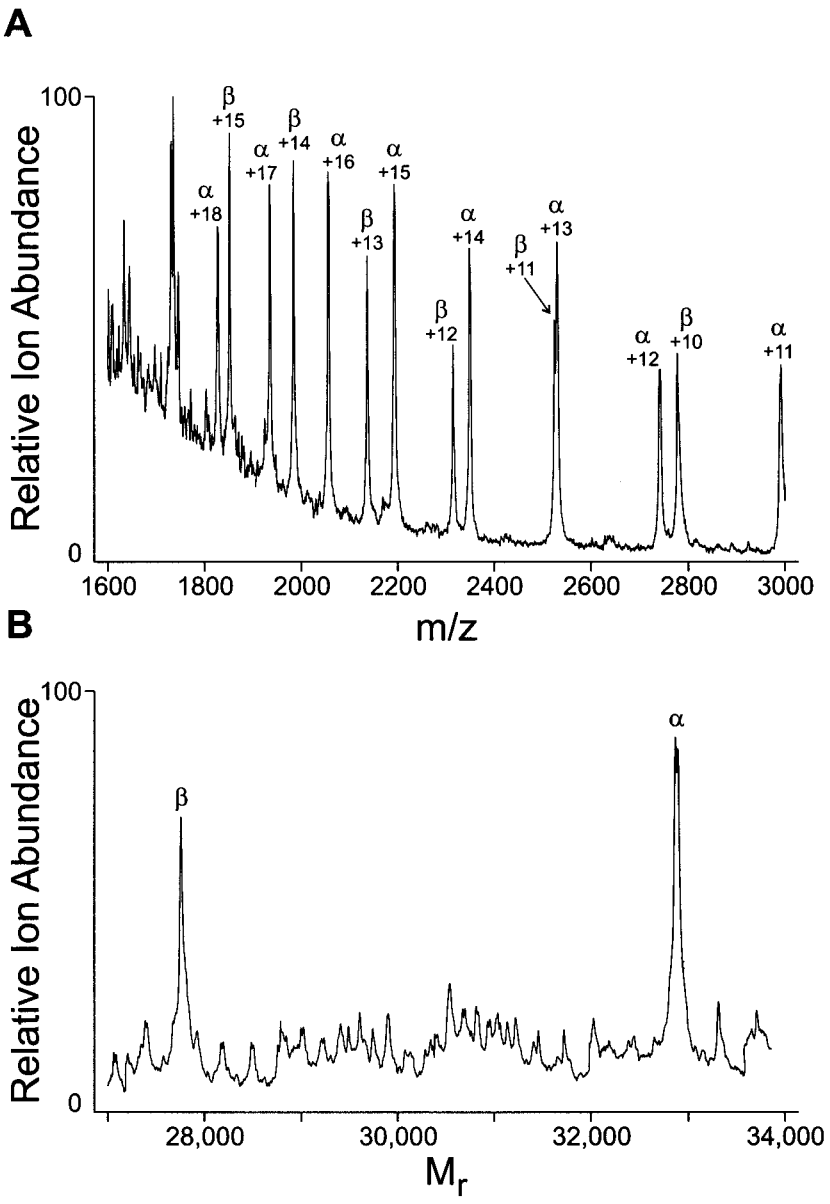

FIG. 5. Porcine ETF denatured in acetonitrile and acetic acid. (A) Multiply charged positive ion. (B) Transformed μESI-MS spectra of denatured porcine ETF. Sample was prepared in 10 mM NH_4HCO_3 pH 8.0, 60% acetonitrile, and 1% acetic acid. The charged states are numerically labeled; α-subunit (α), β-subunit (β).

treated in the same manner (Fig. 4) revealed cation adducted intact α-subunit (33,305 Da) and β-subunit (27,665 Da), with the only cofactor association detectable being β-subunit with AMP (28,011 Da) as shown in Fig. 4B. It should be noted that in both Figs. 3B and 4B, unlabeled "ion" responses are actually artifacts of the transformation algorithm.

Lastly, pETF and hETF heterodimer complexes were completely denatured in the presence of 60:40:1 H_2O: CH_3CN:AcOH. Only two ion series are observed for both pETF (Fig. 5A) and hETF (Fig. 6A). Transformation of the ion series provided the first accurate molecular mass determination for the porcine α- subunit (32,866 Da) and β-subunit (27,756 Da) as shown in Fig. 5B. The human α-subunit and β-subunit M_r values were 33,230 Da and 27,628 Da respectively. These values are in agreement with the expected masses based on the expression plasmid sequence and amino-terminal sequencing data (α- subunit 33,224 and β-subunit 27,624).

DISCUSSION

In this work, the solution chemistry of the ETF holoprotein was investigated using μESI-MS. Under non-denaturing μESI-MS conditions, intact ETF holopro-

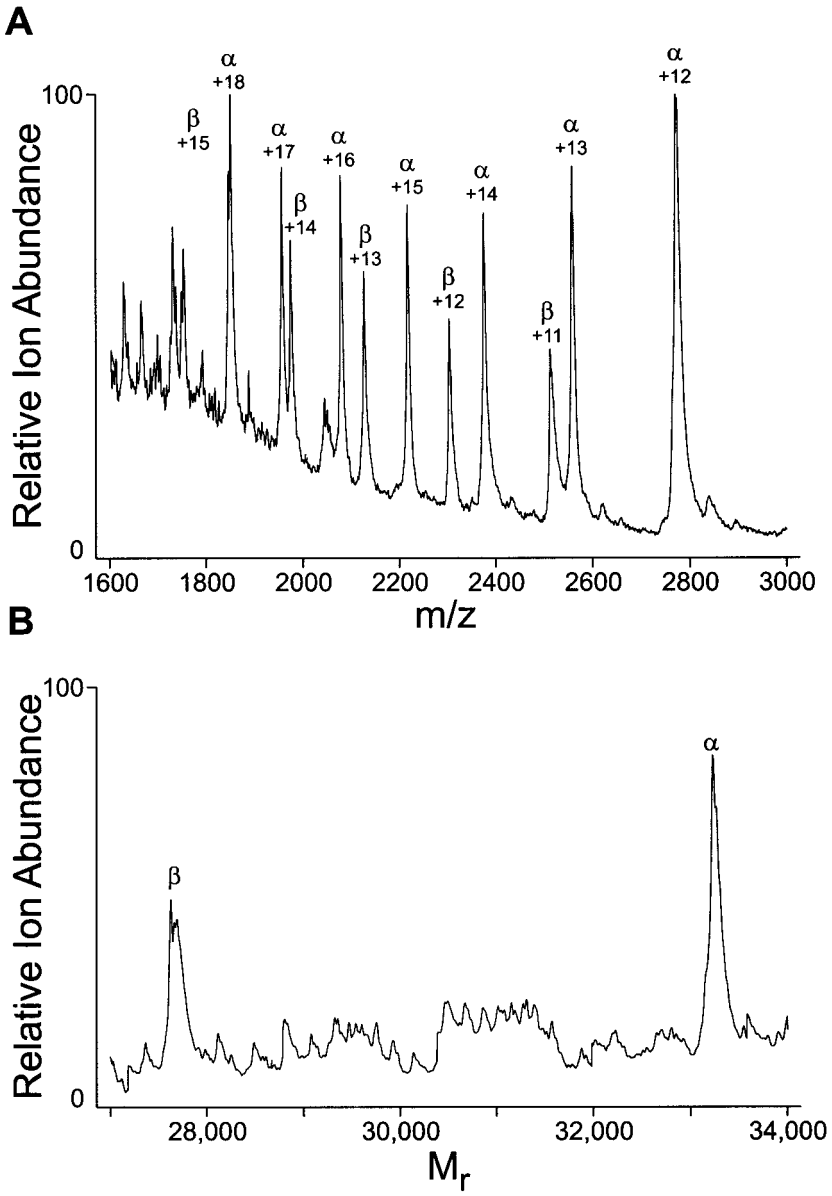

FIG. 6. Human ETF denatured in acetonitrile and acetic acid. (A) Multiply charged positive ion. (B) Transformed μESI-MS spectra of denatured human ETF. Sample was prepared in 10 mM NH_4HCO_3 pH 8.0, 60% acetonitrile, and 1% acetic acid. The charged states are numerically labeled; α-subunit (α), β-subunit (β).

tein was the only species observed, indicating a stable complex of subunits and cofactors that is not gas-phase labile. The transformed mass spectrum of the hETF holoprotein revealed a M_r larger than predicted from the published cDNA sequence. The coding sequences for the α- and β-subunits in the expression plasmid are joined by overlapping initiation and termination codons respectively. Two tandem initiation codons are present for the α-subunit and transcription initiates from the first initiation codon, producing an amino terminus with an additional methionine. The amino-terminal methionine for the β-subunit precedes an alanine, a strong bacterial cleavage signal. The methionine is cleaved during protein production, indicated by an alanine in the first round of amino-terminal peptide sequencing (data not shown). These amino acid changes and adduction of cations explain the observed differences in molecular mass.

Disruption of the ETF holoproteins by sCID yielded relative binding affinities of the cofactors. The AMP molecule readily dissociated from both pETF and hETF holoprotein while FAD remained intact, contradicting predictions based on crystal structure considerations. In the crystal structure, the FAD is located in a solvent accessible crevice formed mainly by the α-subunit with the isoalloxazine ring facing the β-subunit. In contrast,

the AMP is buried deeply within the β-subunit, and on first inspection does not appear to be readily amenable to dissociating from the protein. However, based on the μESI-MS analyses of both pETF and hETF, AMP is lost as the NH_4^+ salt. Similarly, if a small amount of K^+ salt (KCl) is added into the protein solution prior to μESI spraying, AMP is lost as the K^+ salt. In contrast, if a sterically bulky triethylamine salt is added, AMP alone is lost. All this data indicates that there is a channel from the surface readily exposing the AMP to bulk solvent conditions. Closer inspection of the X-ray structure reveals that there is indeed a channel from the AMP site to the protein surface formed by 1 Gln-70, Ala-69, Pro-68, Po-93, Ala-96, Glu-97, Asp-129, Asp-82, His-36, Arg-12 and Lys-11. To our knowledge, the existence of this channel has not been described or commented on previously. Certainly, the role of AMP in ETF has not been defined nor delineated. It is not required for electron transfer, but it thought to be important for rapid and proper folding of the ETF holoprotein (7). Hence, this hydrophilic solvent channel, while allowing relative facile loss of AMP from the protein, may offer further insight into the role of this cofactor in the ETF protein family.

Analysis of the monomeric species after protein complex disruption in methanol revealed notable differences in cofactor associations between human and porcine ETF. The individual subunits lacking cofactor association and the β-subunit with AMP were observed for both, in agreement with crystal structure predictions. However, the AMP association with the β-subunit contrasts the observed sCID of AMP from the holoprotein. The pETF spectrum displayed series representing the β-subunit with FAD and β-subunit with both cofactors. This observation suggests that the β-subunit in pETF has a more intimate association with the FAD than predicted from the hETF crystal structure. The FAD has several interactions with amino acids from the α-subunit and only limited van der Waals interaction with the β-subunit in the crystal structure. The difference in FAD association between the human and porcine ETF is not explained by differences in flavin amino acids. The flavin peptides are highly conserved from bacteria to human (22), and the porcine flavin amino acids that have been sequenced are also highly conserved (unpublished data). The denatured pETF spectra revealed only the individual monomers, verifying noncovalent association of cofactors.

Differences between porcine and human ETF cofactor/subunit interactions are observed using μESI-MS and offer additional insight to predictions based on the X-ray crystallography data. AMP dissociates more readily than the FAD in both pETF and hETF, suggesting a weaker association of AMP. The β-subunit in pETF has a more intimate association with FAD than in hETF and may provide a basis for observed differences in fluorescence. μESI-MS is a powerful emerging tool for investigation of noncovalent protein–protein and protein–cofactor interactions. If affords a new tool for the rapid analysis of such interactions using only μmole-nanomole amounts of protein and provides complimentary data to NMR, X-ray crystallography, and optical spectroscopic studies of noncovalent interactions.

ACKNOWLEDGMENTS

We thank Mrs. Diana Ayerhart for her help in preparing the manuscript and the Mayo Protein Core for amino-terminal protein sequencing. Financial support was provided by the Barbara Woodward Lipps Fund from the Mayo Foundation (J.V.) and PHS Grant RO1-DK45482 (J.V.).

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