*Articles*

# Direct Sequence Data from Heterogeneous Creatine Kinase (43 kDa) by High-Resolution Tandem Mass Spectrometry†

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ABSTRACT: Isoelectric focusing separation of recombinant rabbit muscle creatine kinase (CK) and its $^{282}$Cys → $^{282}$Ser mutant shows the presence of three and two isoforms, respectively, that exhibit equivalent enzymatic activity. Electrospray ionization coupled with Fourier-transform mass spectrometry ($10^5$ resolving power) of both CKs indicates that their major components are within ±2 Da of the $M_r$ value predicted from the cDNA sequences of these mixtures. Dissociation of $(M + nH)^{n+}$ gives no evidence that the components of either CK are isomers; the masses of the 51 fragment ions correlate completely (±1 Da) with the values predicted from the cDNA sequence and confirm the identities of 21 of the 380 amino acids and the $^{282}$Cys → $^{282}$Ser replacement in the mutant. The results are consistent with one or two steps of post-translational amidation/deamidation ($NH_2$ → OH, 16 Da → 17 Da), each of which would produce only a 1 Da difference in $M_r$, with the fragment masses indicating that at least one modification occurs between residues 212 and 282.

Creatine kinase (CK, monomer)[1] (E.C. 2.7.3.2) is a key enzyme in energy metabolism for brain and muscle cells (Watts, 1973; Kenyon & Reed, 1983). Although extensive mechanistic studies of its structure and enzymatic function have been carried out, their significance is in question because of sample purity problems, which have also hampered elucidation of a high-resolution crystal structure (Hershenson et al., 1986). For example, although SDS polyacrylamide gel electrophoresis indicates that the recombinant rabbit muscle (wild type) CK (the isozyme used in most studies) (Kenyon & Reed, 1983) is >99% homogeneous, isoelectric focusing (IEF) separation of it and its C282S mutant still shows three and two bands, respectively (Figure 1), that are of equivalent enzymatic activity (Chen et al., 1990, 1991). This is also observed in the tissue-purified samples. Although many explanations have been advanced (Chen et al., 1991, and references therein), the cause of this heterogeneity has not been identified using DNA sequence data, which yielded only a single sequence (Putney et al., 1984; Chen et al., 1991), or special techniques such as proteolysis−tandem mass spectrometry (MS/MS) (Buechter, 1990). Although the latter technique has been impressively successful in solving many similar problems (Biemann & Papayannopoulos, 1994), in deriving component molecular weights for mixtures of such large proteins from the masses of proteolysis fragments one would face unusual ambiguities. In this report, the whole molecules are examined with electrospray ionization (ESI) (Fenn et al., 1990; Smith et al., 1991) combined with Fourier-transform (FT) MS, (Comisarow & Marshall, 1974; Marshall & Grosshans, 1991) whose high-resolution and MS/MS capabilities have yielded accurate $M_r$ and sequence data for well-characterized proteins (McLafferty, 1994; Senko & McLafferty, 1994) as large as 67 kDa (Speir et al., 1995). This and a study of thiaminase I (42 kDa) (Kelleher et al., 1995a,b) are the first to report

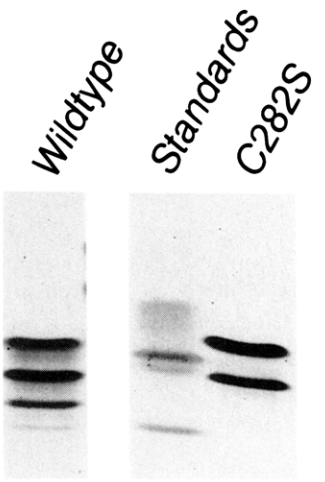


FIGURE 1: Isoelectric focusing gel of wild-type muscle CK and the C282S mutant.

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[1] Abbreviations: CK, creatine kinase; $M_r$, relative molecular weight; ESI, electrospray ionization; FT, Fourier-transform; MS, mass spectrometry; IEF, isoelectric focusing; NS, nozzle/skimmer; rf, radio frequency; SORI/CAD, sustained off-resonance irradiation/collisionally-activated dissociation; $b_n$ and $y_n$ ions, N- and C-terminal, respectively, fragment ions containing $n$-amino acids formed by cleavage of an amide bond of the molecular ion.

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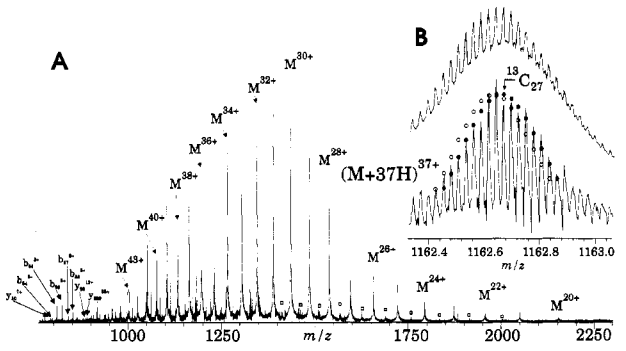


FIGURE 2: (A) ESI/FTMS mass spectrum of wild-type rabbit muscle CK; NS (100 V), sum of 20 scans: □, dimer ions. (B) Expanded $m/z$ scale, resolving power ~$10^5$. Bottom: $(M + 37H)^{37+}$ data plus isotopic distributions predicted from (○) the cDNA sequence and from (●) an equimolar mixture of unmodified, once-, and twice-deamidated enzymes. Top: charge states 19+ to 43+ combined by geometric deconvolution (Mann et al., 1989).

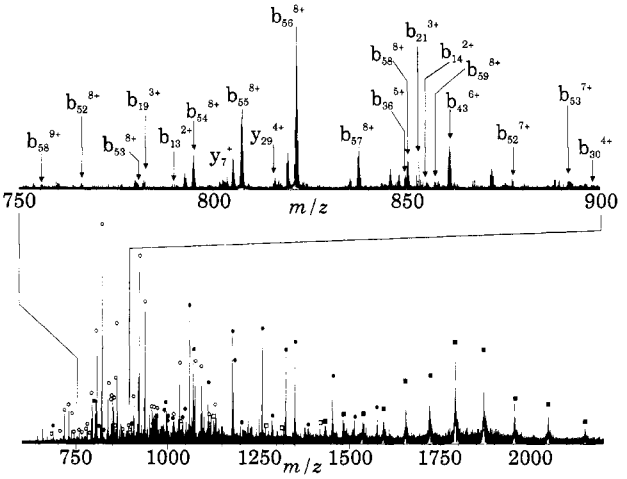


FIGURE 3: NS (200 V), ESI/FTMS mass spectrum of rabbit muscle CK, sum of 25 scans: ■, $(M + nH)^{n+}$; ○, b-type (N-terminal) fragments; ●, y-type (C-terminal) fragments; □, internal fragments.

important new structural data from high-resolution MS of such large biomolecules without prior chemical or enzymatic degradation.

## EXPERIMENTAL PROCEDURES

The CK samples were prepared and isolated by published methods (Chen et al., 1991) except that the cells were lysed by sonication and centrifuged at 48 000$g$ for 30 min. Samples (10 $\mu$g) were electrophoresed using a pH 3−9 gradient gel (5% Novex, San Diego, CA) for 1 h each at 100, 200, and 500 V, fixed in 10% trichloroacetic acid/5% sulfosalicylic acid for 15 min, washed (10% isopropanol/10% acetic acid), and stained in 0.02% Coomassie Blue/0.1% $CuSO_4$/10% isopropanol/10% acetic acid for 1 h. CK (~20 $\mu$M in 76:22:2 $CH_3OH/H_2O/HOAc$) by volume was continuously delivered at a 0.5−1 $\mu$L/min flow rate and electrosprayed from a needle at 2.6−2.9 kV through a metal capillary (~110 °C) and three rf-only quadrupoles into an FTMS cell ($10^{-9}$ Torr) in a modified 6.1 T Millipore-Extrel FTMS-2000 (Beu et al., 1993). Ion accumulation (5 s) in the FTMS cell was achieved with 4.5 and 5.5 V, respectively, on the source and analyzer traps while pulsing in $N_2$ gas ($10^{-6}$ Torr). After 60−120 s to pump out the gas pulse, ion measurement was performed in the direct mode (512K data points, bandwidth 100, 125, or 150 kHz). Ion fragmentation in the electrospray source was effected with a potential difference between the input nozzle and exit skimmer (NS) in the ~1 Torr pressure region (Loo et al., 1991; O'Connor et al., 1995). Alternatively, ions trapped in the cell were subjected to sustained off-resonance irradiation (SORI)/collisionally-activated dissociation (Gauthier et al., 1991; Senko et al., 1994) using 1 s rf excitation pulses ±1.8 kHz off-resonance (~5 $V_{pp}$) with $N_2$ collision gas ($10^{-6}$ Torr). All mass values are for the most abundant isotopic peak (Senko et al., 1995), which differs from the isotopically-averaged $M_r$ value by <0.3 Da (McLafferty, 1994).

## RESULTS AND DISCUSSION

Separation of the wild-type CK on a 5% IEF gel yields (Figure 1) several isoforms: p$I$ values of 7.3, 7.1, and 6.9, with an additional faint band at 6.7. A time-dependent increase in the 6.7 isoform is also observed (data not shown). The mass spectrum (Figure 2A) of heterogeneous rabbit muscle CK chiefly shows intact $(M + nH)^{n+}$ peaks and little fragmentation. Fitting the resolved isotopic peak abundances to those expected theoretically (dots, Figure 2B) indicates that all three major components seen in the IEF gel of Figure 1 must be within 2 Da of the same $M_r$ value 42 982 ($^{13}C_{27}$, averaging $z$ = +19 to +43); a value of 42 981 ($^{13}C_{27}$) is expected from the cDNA sequence (Putney et al., 1984; Chen et al., 1991). Although a modest (100 V) NS potential was used to dissociate molecular ions of functional $\alpha_2$ homodimeric rabbit muscle CK ($M_r \approx$ 86 000), such ions can also be observed at odd charge states (e.g., 49+, 51+, 53+, etc.; Figure 2A) and between the monomeric isotope peaks (Figure 2B).

IEF separation of the C282S CK generated by site-directed mutagenesis shows (Figure 1) isoforms of p$I$ 7.3 and 7.1. These must also have near-identical $M_r$ values, 42 967 ± 2, which agrees well with that expected from the sequence (Cys → Ser; −SH → −OH, −16 Da).

The nearly identical $M_r$ values for the isoforms of the two mixtures eliminate explanations for heterogeneity such as proteolysis or oxidation of methionine or cysteine (Chen et al., 1991, and references therein). It is possible that the observed heterogeneous enzyme isoforms are isomers in which the positions of one or more amino acids are transposed. Thus dissociation of the isomers should produce fragments of different masses (e.g., ABCD + ACBD → AB + AC + CD + BD). Nozzle/skimmer (NS) dissociation (Loo et al., 1991; O'Connor et al., 1995) products are visible in Figure 2A, despite the incomplete dissociation of the non-covalent dimer ions. Increasing the NS potential (200 V, Figure 3) yields many more fragments; their masses plus those from sustained off-resonance irradiation/collisionally-activated dissociation (Gauthier et al., 1991; Senko et al., 1994) of the isolated +28 charge state (its relative abundance enhanced vs Figure 2 by optimizing ion-trapping parameters during ESI) are tabulated in Figure 4 vs the cDNA sequence data (Putney et al., 1984). Mass differences (outside columns, Figure 4) confirm the positions of 21 amino acids (including a 10-residue sequence, sufficient for design of an oligonucleotide probe) (Putney et al., 1984). Mass sums of two pairs [13 729 ($^{13}C_8$) + 29 254 ($^{13}C_{19}$) = 42 983 and 24 087 ($^{13}C_{15}$) + 18 895 ($^{13}C_{12}$) = 42 982] agree with the measured $M_r$ value, and the last 18 895 fragment mass

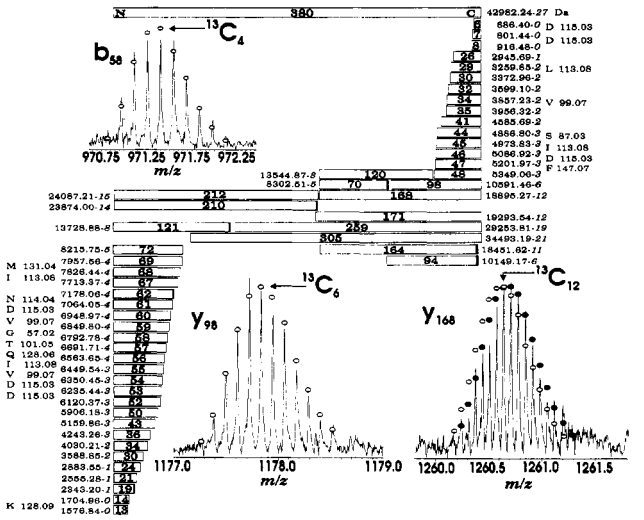


FIGURE 4: NS and SORI spectral data (fragment mass values for $^{13}C_n$ peak, $n$ value italicized) of rabbit muscle CK, correlated with the cDNA sequence (Putney et al., 1984; Chen et al., 1991) and Pro positions (heavy lines, N-terminal to Pro cleavages). Horizontal boxes on the left and right represent b-type and y-type fragments, respectively (those in between are internal fragments), with the numeral representing the number of amino acid residues in the fragment. External columns: expected mass differences for indicated amino acids. Insets: isotopic distributions of key fragment ions per Figure 2 legend.

corresponds to the pairs 8303 ($^{13}C_5$) + 10 591 ($^{13}C_6$) and 13 545 ($^{13}C_8$) + 5349 ($^{13}C_3$). Thus, the Figure 4 data correlate completely (±1 Da) with the cDNA sequence of the heterogeneous rabbit muscle CK (Putney et al., 1984; Chen et al., 1991), restricting potential amino acid exchanges in the three components to regions between the cleavage locations shown (the longest region has 88 of the 380 total amino acids). The 20 fragment masses of the NS (100 V) ESI/FTMS mass spectrum of the C282S mutant are quite similar to those of the corresponding spectrum of the wild-type protein, with a −16 Da shift observed only for the fragments containing Ser-282 ($y_{168}$ is the shortest).[2]

These results show conclusively that the observed CK heterogeneity is not due to various post-translational modification processes (Chen et al., 1991, and references therein). On the other hand, the results are completely consistent with another proposal (Chen et al., 1991, and references therein) wherein the first isoform is composed only of the amino acids from the cDNA sequence reported (Putney et al., 1984; Chen et al., 1991), and the others are modified with one and/or with two deamidations, each of which produces a 1 Da increase ($NH_2$ → OH, 16 Da → 17 Da). The calculated 1 Da increase in the average $M_r$ of this mixture vs the cDNA predicted value, agrees well with the experimental results (Figure 2B). The more accurate masses for the $b_{58}$ and $y_{98}$ ions (Figure 4) agree with the cDNA values, suggesting that the major deamidation sites are in the 224 residues (of the 380 total) after Gly-58. Although experimental $m/z$ values for the isotopic peaks of the reported fragments provide charge state ($z$), and thus $m$, which are both assignments of high confidence, the accuracy of the measured abundances of the isotopic peaks are more sensitive to random statistical fluctuations of ion populations, making the assignment of the most abundant isotopic peak less rigorous for low-abundance fragments (McLafferty, 1994). The $y_{168}$ value appears to be high by 1 Da, but this is at the limit of present accuracy; this value (18 895), if correct, would restrict at least one major deamidation site (and possibly both) to a 70-residue span between Asp-212 and Cys-282. This region is relatively rich in Asn (five of 16); two of these (Asn-219 and Asn-229) are conserved in all CKs sequenced to date, suggesting they are relatively important for CK structure and/or function. Errors of translation due to tRNA−mRNA mismatches can also account for heterogeneity at certain amino acid positions (Schimmel, 1989). For example, AA-(U/C) encodes Asn and GA(U/C) encodes Asp. Likewise, CA(A/G) encodes Gln and GA(A/G) encodes Glu. Misalignment of charged tRNA with the mRNA could result in an Asn → Asp or Gln → Glu substitution in the resultant protein. However, these mismatches cannot account for the observed time-dependent increase in the p$I$ 6.7 isoform. This is consistent with chemical deamidation; additional bands appear in the IEF gels with increasing time after isolation and storage *in vitro* (Chen et al., 1995). Current experiments utilizing the isolation and analysis of single IEF bands and isotopic labeling with $H_2{}^{18}O$ could more accurately locate the Asn → Asp and/or Gln → Glu exchanges in the primary structure of CK to differentiate chemical deamidation from translational misreading of the CK mRNA.

The results shown here represent the summation of three NS and two collisional dissociation experiments (50−100 pmol consumed per experiment). However, recent advances in ESI techniques in this and other laboratories using gold-coated capillary electrodes (Wilm & Mann, 1994; Valaskovic et al., 1995) have reduced ESI sample requirements to the femtomole level. Although acquisition of such ESI/FTMS spectra here requires less than 1 h (including tuning), data analysis required several days of human effort. However, experience and automation of data analysis have already reduced this effort substantially for new problems.

[2] In the mutant spectrum, peaks formed by $^{282}$Ser−$^{283}$Pro bond cleavage were not present; gas-phase H transfer from the SH group of Cys may be more favorable than that from the OH group of Ser.

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