ACCELERATED COMMUNICATION

Chemical-modification rescue assessed by mass spectrometry demonstrates that γ-thia-lysine yields the same activity as lysine in aldolase

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Abstract

The role of active site residues in fructose 1,6-bisphosphate aldolase is investigated by chemical-modification rescue. An active-site mutation, K107C, is constructed in a background where the four solvent-accessible cysteine residues are converted to alanine. The resulting mutant, tetK107C, when reacted with bromoethylamine (BrEA), shows a 40-fold increase in activity (to 80% that of wild type). Determination of the sites and their degree of modification using electrospray ionization Fourier transform mass spectrometry (ESI-FTMS) is developed, allowing correlation of activity after chemical modification rescue to the degree of modification. The stoichiometry of the reaction is 2.5 aminoethylations per subunit, as measured by ESI-FTMS. Protein modification with a double-labeled mix (1:1) of natural abundance isotope (d_0-BrEA) and 2-bromoethyl-1,1,2,2-d4-amine hydrobromide (d_4-BrEA), followed by dialysis and trypsin digestion, shows aminoethylated peptides as "twin peptides" separated by four mass units in ESI-FTMS analysis. Using this detection procedure under nondenaturing (native) conditions, C107 is aminoethylated, whereas the four buried thiols remain unlabeled. Aminoethylation of other residues is observed, and correlates with those peptides containing histidine, methionine, and/or the amino terminus. Quantification of the aminoethylation reaction is achieved by labeling with nondeuterated d_0-BrEA under denaturing conditions following double labeling under native conditions. In addition to complete labeling all five thiols, the intensity of the d_0-BrEA peak for C107 containing peptides increases, and the change in the d_0/d_4 ratio between native and denaturing conditions shows 82 ± 4.5% aminoethylation at C107. This correlation of modification with the recovered activity, indicates that γ-thia-lysine replaces lysine in the catalytic mechanism. Kinetic constants measured for the rescued K107C mutant enzyme with the substrates fructose 1-phosphate and fructose 1,6-bisphosphate are consistent with the role of the positively charged lysine binding to the C6-phosphate. ESI-FTMS, combined with this double-labeling procedure, allows precise identification of sites and measurement of degree of protein modification.

Keywords: Aldolase; chemical modification rescue; bromoethylamine; mass spectrometry; enzyme mechanism; protein modification; alkylation; cysteine; lysine

Insight into the function of catalytic groups at the active site of enzymes has been obtained by chemical modification rescue experiments (Benkovic et al. 1972; Smiley and Jones 1992; Kim et al. 1994; Highbarger et al. 1996; Nash et al. 1997; Bochar et al. 1999). However, separation of effects of

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incomplete modification from the effects of the substitution has not been achieved. Smith and Hartman (1988) rescued an inactive K329C mutant of ribulose bisphosphate carboxylase/oxygenase by modification with bromo-ethylamine (BrEA) to form the analog of lysine, γ-thia-lysine. Aminoethylation of the mutant enzyme restored activity to 30% of the wild type (wt). Aminoethylation of wt, however, resulted in 30% inhibition, leading to the speculation that an inhibitory modification of an endogenous cysteine occurred elsewhere in the protein. Planas and Kirsch (1991) performed aminoethylation of a K258C mutant of aspartate aminotransferase, which resulted in 2% wt activity. When the rescue experiment was performed on a K258C mutant enzyme wherein all five endogenous cysteines were substituted by alanine (Gloss and Kirsch 1995), 10% of wt activity was observed. Clearly, undesirable reaction at endogenous thiols or other nucleophiles is a concern in chemical modification rescue with BrEA. Additional insight can be gained by the determination of the sites and level of modification.

Chemical modification rescue has not been reported for class I aldolases, which have three important active-site lysines (K107, K146, and K229) that are necessary for cleavage of fructose-1,6-bisphosphate (Fru1,6P2) into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Grazi et al. 1962; Morris and Tolan 1994; Wang et al. 1996). One crystal structure of aldolase with a noncovalently bound Fru1,6P2 showed that the C6-phosphate of the substrate was within 3 Å of the ε-amino group of K107 (Dalby et al. 1999). Consistent with a phosphate-binding role, the k_{cat}/K_m of a K107A mutant toward Fru1,6P2 was 500-fold reduced (Wang et al. 1996), but was unchanged with fructose-1-phosphate (Fru1P) (Choi et al. 1999). These results suggest that the ε-amino group of K107 binds the C6-phosphate of the Fru1,6P2; however, the loss of activity due to mutagenesis cannot rule out any indirect effects. Thus, this important binding residue was targeted for chemical modification rescue to investigate its role through a gain in function.

Interpreting data from chemical modification rescue experiments is augmented by measurement of the specificity and efficiency of the modification reaction. Mass spectrometry is a powerful method for determining the sites of chemically induced or physiologically induced protein modifications (Resing et al. 1995; Chen et al. 1999; Oda et al. 1999; James 2000; Patterson 2000). For instance, in a proteomic study, Gygi et al. (1999) employed thiol-specific, isotope-coded affinity tags to quantitatively analyze changes in protein expression. *Saccharomyces cerevisiae* were grown on either ethanol or galactose as a carbon source, and total protein was labeled with either a deuterated or nondeuterated affinity tag, respectively. By combining the preparations and performing LC-MS, the differences in protein expression were observed and quantified.

In the present study, a mass spectrometric approach was used to solve the problem of identifying and quantifying sites of chemical modification with BrEA in a K107C mutant of aldolase. Aminoethylation resulted in recovery of 80% of wt activity. The question arises as to whether this site was 80% modified or completely modified, but the γ-thia-lysine was only 80% as effective as lysine at this position. Electrospray-ionization Fourier-transform mass spectrometry (ESI-FTMS) was used to detect the amount of aminoethylation per enzyme monomer. All sites of modification were determined by utilizing a double-labeling strategy with two isotopic forms of BrEA, Br-CH_2-CH_2-NH_3^+ (d_0-BrEA) and Br-CD_2-CD_2-NH_3^+ (d_4-BrEA), followed by trypsin digestion and ESI-FTMS identification. The degree of modification was measured at each site after double-labeling protein with d_0/d_4-BrEA (1:1) under nondenaturing (native) conditions followed by labeling under denaturing conditions with d_0-BrEA. Normalizing to the peak of the d_4-BrEA–modified peptide, the relative increase in the d_0-BrEA peak revealed the amounts of reacted and unreacted thiol present under native conditions. The 80% wt activity restored in a K107C mutant enzyme correlated with 80% modification at C107. Furthermore, the high sensitivity of ESI-FT-MS enabled detection of other low-level modifications at sites other than cysteine.

Results

Chemical modification rescue

Reaction with nontarget cysteine residues was limited by mutating the codons for four solvent-exposed cysteines (C72, C239, C289, and C338; Lai et al. 1971) to alanine codons. The resulting substituted protein, gtet, exhibited wt kinetics (Table 1). A K107C substitution constructed in the background gtet (tetK107C) resulted in a 700-fold drop in k_{cat}/K_m, similar to that in a K107A-substituted enzyme in wt background (Wang et al. 1996). When tetK107C was treated with BrEA for 8 h, the modified protein, tetK107C–EA, recovered 80% of gtet–EA specific activity (Fig. 1A). The k_{cat} measured with enzyme after 20-h incubation was 82 ± 2 % that of gtet-EA. The k_{cat}/K_m of gtet–EA did not significantly differ from that of similarly treated wt (Table 1). In addition, the specific activity of tetK107C toward Fru1P was only fourfold lower than gtet and regained wild-type activity after modification (tetK107C–EA).

Amount and sites of modification

Relative to the spectra obtained for unmodified tetK107C, ESI-FTMS of tetK107C–EA demonstrated the occurrence of multiple modifications (Fig. 1B–D). The deconvoluted spectrum showed that all protein was modified, and that the degree of aminoethylation ranged from one to five amino-

Table 1. *Activity of aldolase and mutants*[a]

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
wt	14.0 ± 0.3	14 ± 1	1000 ± 70
gtet	14.0 ± 1	12 ± 0.5	1170 ± 90
tetK107C	0.76 ± 0.02	450 ± 10	1.7 ± 0.05
K107A[b]	0.24 ± 0.01	170 ± 12	1.4 ± 0.1
tetK107C-EA[c]	10.8 ± 0.1	10.7 ± 0.2	1010 ± 20
gtet-EA[c]	13.1 ± 0.1	16.0 ± 0.4	820 ± 20

[a] For wt, gtet, gtet-EA, and tetK107C-EA, 0.2 μg of protein was used and Fru1,6P2 ranged from 0.94 to 120 μM. For tetK107C, 4 μg of protein was used and Fru1,6P2 ranged from 4.2 to 540 μM.
[b] Previously published data (Wang et al. 1996).
[c] Enzyme after 20-h reaction with BrEA.

ethylations per monomer (Δm = 43.066 u, aminoethylation versus H). The sites of modification were determined by ESI-FTMS of the tryptic digests of tetK107C and tetK107C–EA. The mass spectrum of the digest of tetK107C–EA is shown in Figure 2 for which 70% of 114 isotope-resolved peaks were assigned by correlating observed masses to calculated masses (Table 2, only the most abundant charge-state given). Observed masses were derived by determining the charge state from the baseline-resolved differences in isotope-peak spacing. Nearly complete coverage of the protein was obtained (Fig. 3) except for two amino-acid positions (K139 and K317) occurring at the end of lysine repeats.

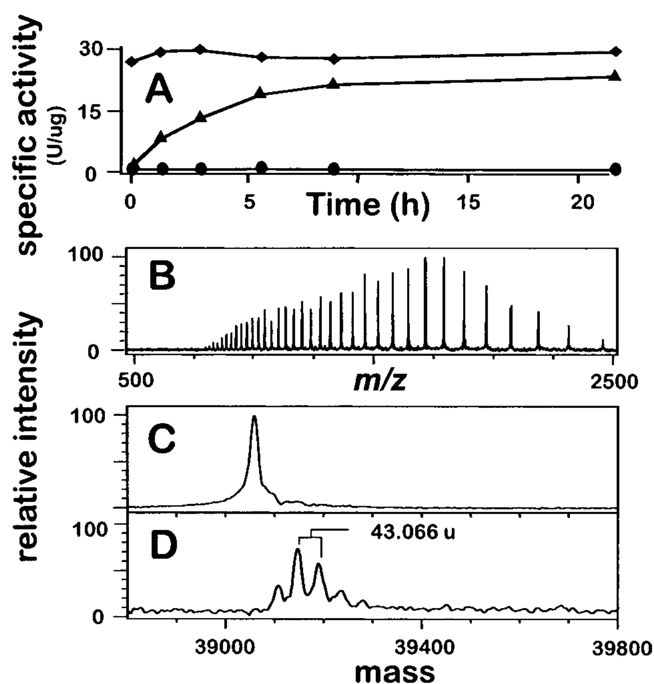

Fig. 1. Chemical modification rescue of tetK107C. (*A*) Modification of tetK107C with BrEA monitored by recovery of specific activity in units aldolase activity/mg. Conditions: 50 mM BrEA, 200 mM AMPSO, pH 8.9, 0.01 mM protein, 25°C. (●), Unreacted tetK107C; (▲), tetK107C–EA; (♦), gtet–EA. (*B*) Fourier-transform spectrum of ESI-FTMS of unreacted tetK107C. (*C*) Deconvolution spectrum of (*B*). (*D*) Deconvolution spectrum of aminoethylated tetK107C–EA. Modification conditions: 50 mM BrEA, 200 mM Tris-Cl, pH 8.9, 20°C, 20 h. Protein samples introduced into the mass spectrometer were 20 μM aldolase in 100 mM NH_4OAc/1% formic acid.

Partial deamination for two peptides, T_{15} and T_{60}, was apparent in the baseline-resolved peaks from the ESI-FTMS generated spectra (Δm = 1 u, amide versus hydroxyl) (Table 2). For these peptides the isotopically-resolved peaks occurred in a distribution that was wider than predicted. The one mass unit difference is consistent with deaminated residues reported in the aldolase protein sequence (Tolan et al. 1984). For instance, Asn-119, which is one of two amide residues in T15, was observed as aspartate in protein sequence determination (Lai et al. 1974).

Employing a double-labeling strategy (1:1 mixture of d_0-BrEA and d_4-BrEA), the detection of two sets of isotope-resolved peaks separated by four mass units easily identified the modification sites. Figure 4A shows that at the T_{14}–(d_0)–EA and T_{14}–(d_4)–EA the peak sets are separated by 2.018 on the *m/z* axis. At a 2+ charge state, this separation correlated with the 4.03 mass unit difference expected after double labeling. In this way, seven modified peptides (T_1, T_3, T_{14}/T_{51}, T_{21}, T_{26}, and T_{27}) were observed (Table 3). Of these, only T_{14} and T_{51} contained cysteine (C107). Peptides of masses corresponding to unmodified forms of T_{14} and T_{51} were not observed, suggesting the peptide fragment was present mostly in the modified form. When labeling was performed on denatured protein, peptides containing internal cysteines (T_{16}, T_{20}, T_{24}, and T_{60}) became modified. By performing double labeling under native conditions and subsequent labeling under denaturing conditions with only d_0-BrEA, peptides containing internal cysteines were modified only in the d_0 form. For example, Figure 4C–D shows the results of modification of the T_{20} peptide, which contains C149. Under native conditions T_{20} was not modified (Fig. 4C, *m/z* 462.28), but was completely modified under denaturing conditions (Fig. 4D, *m/z* 505.33). Neither the d_4-aminoethylated form (*m/z* 509.34), nor the d_0-aminoethylated form (*m/z* 505.33), was observed under native conditions, which shows that labeling of T_{20} occurred only in denaturing conditions. Similar labeling results were observed for the three other internal cysteine peptides (T_{16}, T_{24}, and T_{60}), indicating the four internal thiols were inaccessible to BrEA modification in the native state. Modification at sites other than cysteine was observed for peptides T_1, T_3/T_{40}, T_{11}/T_{48}, T_{21}, T_{26}, T_{27}/T_{65}, and T_{38} (Table 3). For example, peptides T_1 and T_{21} were clearly modified under native conditions (Fig. 4E), yet they contain no cysteine. Under denaturing conditions (Fig. 4F), the T_1 peptide (PHSHPALTPEQK) was modified completely. For the T_{21} peptide, further modification after denaturation was evident by the increase in the d_0-aminoethylated form, although modification was incomplete.

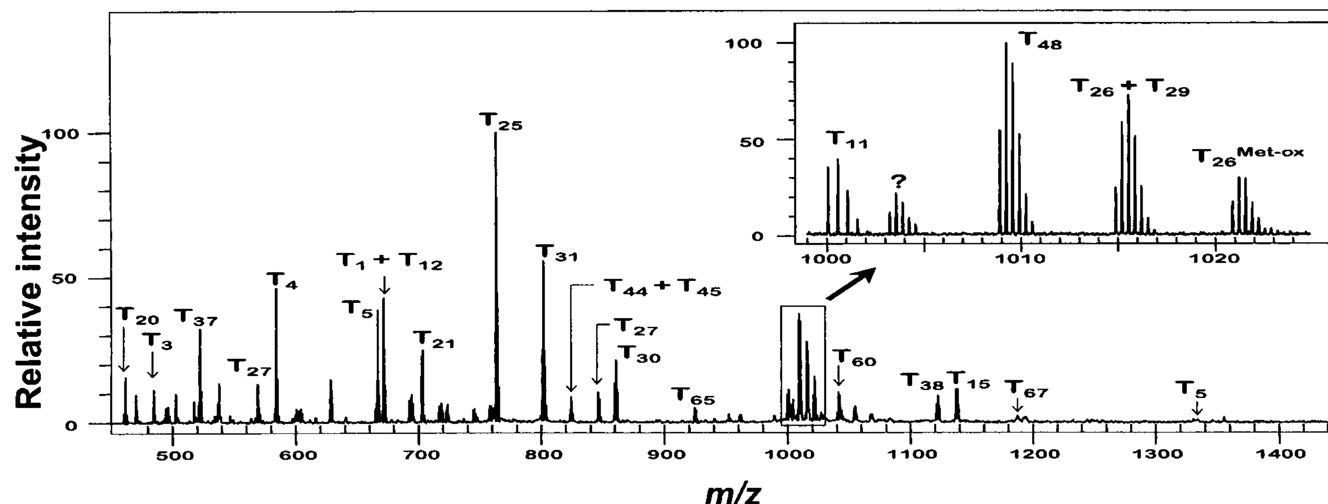

Fig. 2. Mass spectrum of modified, digested tetK107C–EA. ESI-FTMS of tetK107C–EA trypsin peptides double-labeled with 1:1 d_0/d_4-BrEA (100 mM total) as in Figure 1D. For clarity, only major peaks are labeled with assignments defined by the method of Chowdhury et al. (1990). (*Inset*) Overlapping peak sets of T_{26} and T_{29} and methionine oxidation peak, T_{26}-Met-ox (see Table 3). The effective concentration of d_4-BrEA was determined by comparing the rate of activity rescue to that of commercial BrEA (d_0-BrEA) determined at known concentrations.

Quantification

For the peaks exhibiting complete aminoethylation after denaturation, quantification was achieved by measuring the change in d_0/d_4 ratio between the native and denatured forms. For example, the T_{14} peptide was modified under native conditions with a 1:1 mixture of d_0/d_4-BrEA, as observed by the d_0- and d_4-labeled isotope peak sets (Fig. 4A). Under denaturing conditions (Fig. 4B), a measurable increase was observed for the d_0-labeled peaks relative to the d_4-labeled peaks. Because the intensity of d_4-labeled peaks does not change between native and denaturing conditions, these peaks serve as an internal standard for peak intensity normalization. Under native conditions, the ratio of peak intensities of d_0/d_4 for T_{14}–EA was 0.88 (4.10/4.68, Table 3). When denaturing conditions were used, the ratio increased to 1.13 (7.53/6.65). The percentage change in the native versus denatured ratios was 77% ($0.88/1.13 \times 100$, Table 4). Thus, 77% of T14 was modified under native conditions. Similarly, for T_{51}–EA 86% modification was observed. Assuming C107 was the likely site of modification, $82 \pm 5\%$ was modified under native conditions. This corresponded to the amount of activity recovered (81–84 %) after aminoethylation (Table 1, Fig. 1).

Discussion

Previous studies have shown the efficacy of double-labeling strategies with ESI-FTMS in the rapid identification of protein modification sites (Gygi et al. 1999; Goshe et al. 2001). For instance, a suicide inhibitor of thiaminease I, 4-amino-6-chloro-2,5-dimethylpyrimidine was synthesized with either a 2-(d_0)-methyl or a 2-(d_3)-methyl and combined in a 1:2 ratio to facilitate identification of the modified peak in ESI-FTMS of an Asp-N digest (Kelleher et al. 1997). The modified peptide was identified as a set of *m/z* peaks with a 32:68 ratio. In this report, the calculated d_0/d_4 isotope ratio was 50:50, but the observed average ratio was $46{:}54 \pm 1.2\%$, demonstrating that the technique provides a precise measure of the isotope-label ratio. In this report, it is the change in the ratio between peak intensities under native and denaturing conditions that allows accurate quantification of labeling when the chemistry of modification is not limiting. At C107, the change in the labeling ratio demonstrated that the level of modification (77–86%) was similar to the level of recovered activity (81–84%), indicating the lack of complete activity recovery is due to incomplete modification at C107 and not to inhibitory reactions at non-target residues or to a difference in the chemistry of a γ-thia-lysine compared to lysine. Clearly, using the change in d_0/d_4 ratio for quantifying the degree of modification under native conditions requires that the site of modification react completely in the denatured state. For instance, T_{21} has a calculated modification ratio of 55%, yet, as apparent from the two sets of peaks shown in Figure 4F, it remains only partially labeled (ca. 30%) even after reaction under denaturing conditions. These sites were all less reactive than cysteine.

Aminoethylation at sites other than cysteine

In the current study, ESI-FTMS analysis has indicated reaction of BrEA with sites other than cysteine (Table 4). Of these, only T_1 was >90% modified (under native conditions) and thus, along with the target C107, accounts for a majority

Table 2. *Peak assignments of tryptic peptides*[a]

Peptide	Calculated *m/z* (charge)	Native observed *m/z* (intensity)	Denatured observed *m/z* (intensity)
T_1	671.35 (2)	671.37 (1.27)[b]	—
T_3	470.75 (2)	470.76 (7.46)	470.76 (13.09)
T_4	584.38 (1)	584.39 (42.07)	584.39 (45.49)
T_5	666.85 (2)	666.87 (34.50)	666.87 (40.62)
T_7	745.86 (2)	745.88 (3.21)	745.88 (3.29)
T_9	485.25 (1)	485.26 (9.04)	485.26 (10.70)
T_{11}	1000.05 (2)	1000.09 (12.42)	1000.09 (17.84)
T_{12}	671.86 (2)	671.88 (42.22)[b]	671.88 (39.31)[b]
T_{15}	1137.07 (2)	1137.12 (11.91)	1137.12 (19.26)[c]
T_{16}	612.28 (1)	612.29 (<1)	—
T_{18}	723.33 (1)	723.35 (5.05)	723.35 (10.23)
T_{20}	462.28 (1)	462.29 (10.24)	—
T_{21}	703.03 (3)	703.06 (22.92)	703.06 (14.60)
T_{24}	870.40 (1)	870.41 (<1)	—
T_{25}	763.47 (1)	763.49 (100.00)	763.49 (100.00)
T_{26}	1014.85 (3)	1014.91 (11.35)[b]	1014.91 (4.47)[b]
T_{27}	846.42 (2)	846.45 (9.84)	846.45 (13.39)
T_{29}	1015.19 (3)	1015.24 (27.27)[b]	1015.23 (12.49)[b]
T_{30}	860.48 (2)	860.51 (23.09)	860.51 (21.14)
T_{31}	801.48 (1)	801.50 (50.24)	801.50 (83.79)
T_{32}	518.27 (1)	518.29 (5.90)	518.29 (8.96)
T_{34}	503.28 (1)	503.29 (7.53)	503.29 (8.77)
T_{37}	522.29 (2)	522.31 (29.82)	522.31 (29.16)
T_{38}	1121.52 (2)	1121.56 (6.34)	1121.56 (8.70)
T_{40}	534.79 (2)	534.80 (2.50)	534.80 (3.37)
T_{43}	744.90 (2)	744.93 (3.46)	744.93 (2.20)
T_{44} or T_{45}	823.91 (2)	823.93 (8.44)[b]	823.93 (12.26)[b]
T_{48}	1008.88 (3)	1008.93 (18.68)	1008.93 (18.68)
T_{56}	533.26 (2)	533.30 (1.25)	533.30 (1.25)
T_{60}	1040.86 (3)	1040.91 (8.48)[c]	—
T_{65}	924.47 (2)	924.50 (4.25)	924.50 (4.73)
T_{66}	1067.22 (3	1067.26 (1.24)	1067.27 (1.20)
T_{67}	1186.88 (4)	1186.94 (2.47)	1186.94 (<1)
T_{73}	547.28 (2)	547.30 (2.21)	547.30 (3.07)
T_{74}	600.35 (2)	600.36 (4.03)	600.36 (2.47)
T_{81}	601.64 (3)	601.65 (1.93)	601.65 (1.03)

[a] Labeling under native conditions with only the highest intensity *m/z* for each peptide shown.
[b] Peptides with overlapping or isobaric peaks.
[c] Peptides with masses wherein partial deamination would account for the observed masses.

of the modification observed. The identity of the nucleophiles is unclear. Previous reports have shown BrEA may react with methionine (Schroeder et al. 1967), histidine, and/or the amino terminus (Raftery and Cole 1963). In this report, histidine is common to all modified peptides that do not contain cysteine, and thus is the likely site of modification. Furthermore, three peptides (T_{38}, T_{48}, and T_{60}) were aminoethylated at a number of sites greater than the number of histidines and methionines, which may be explained by bis-modification at histidine. Bis-substitution has been observed with other histidine-specific reagents such as diethylpyrocarbonate (Miles 1977).

In conclusion, the gain of near wild-type activity for the chemical-modification rescue of the C107 mutant aldolase shows unequivocally that K107 has a role in C6-phosphate binding at the active site. The C6-phosphate binding site has been alternatively implicated as R303 from X-ray crystallography data (Choi et al. 1999), or K107 from chemical modification (Lai et al. 1974), site-directed mutagenesis (Takasaki et al. 1990; Wang et al. 1996; Choi et al. 1999), and X-ray crystallography data (Dalby et al. 1999). These two residues are 10 Å apart in the crystal structure (Choi et al. 1999; Dalby et al. 1999). A loss of activity toward Fru1,6P2, with minimal effect toward Fru1P (Choi et al. 1999) has been cited as evidence for the C6-phosphate binding role by K107. However, these previous studies could not rule out possible explanations for this preferential loss of activity based on indirect causes such as folding or subtle conformational changes due to the loss of the lysyl-residue. This report clearly shows that modification of C107 to a γ-thia-lysine is responsible for the regained activity, and any indirect effects from site-directed mutagenesis at this site can be ruled out. Furthermore, ESI-FTMS could detect all significant sites of chemical modification. A novel double-labeling strategy, using native and denaturing conditions, quantified the amount of labeling at C107 based on the change in isotope ratio. Undesirable reaction with buried thiols did not occur in aldolase under native conditions, but reaction with residues other than cysteine was apparent. The amount of aminoethylation was in agreement with the level of activity attained, thus implying that steric and/or electronic differences between a "rescued" lysine (γ-thia-lysine) and lysine do not significantly affect the role of the amino acid in the active site. Alternatively, but less likely, γ-thia-lysine may participate in catalysis more effectively than lysine, but the effect is compensated by the negative effect of modification at sites other than cysteine.

Materials and methods

Chemicals

The chemicals used were 2-bromoethylamine hydrobromide (Fluka, Inc.); (1,1-2,2-d_4)-ethanolamine hydrochloride (Cambridge Isotope Laboratories, Inc.); CM-Sepharose Fast Flow (Pharmacia Biotech, Inc.); sequencing-grade modified-trypsin (Roche Diagnostic GmbH). All other chemicals were reagent grade or better.

Plasmids and PCR mutagenesis

The construction of plasmids pPB1 and pPB14 has been described elsewhere (Beernink and Tolan 1992; Morris and Tolan 1993). Briefly, pPB1 is an 'ATG' vector that has pUC19 origin of replication and the *trc* promoter. The pPB14 clone expresses wt rabbit-muscle Fru1,6P2 aldolase in pPB1. Of the eight cysteines present in aldolase, four are solvent accessible (C72, C239, C289, C338) (Lai et al. 1971) and were substituted by alanine in pPB14 by modification of the overlap-extension PCR method, as reviewed by Ling and Robinson (1997). The primers (National Biosciences,

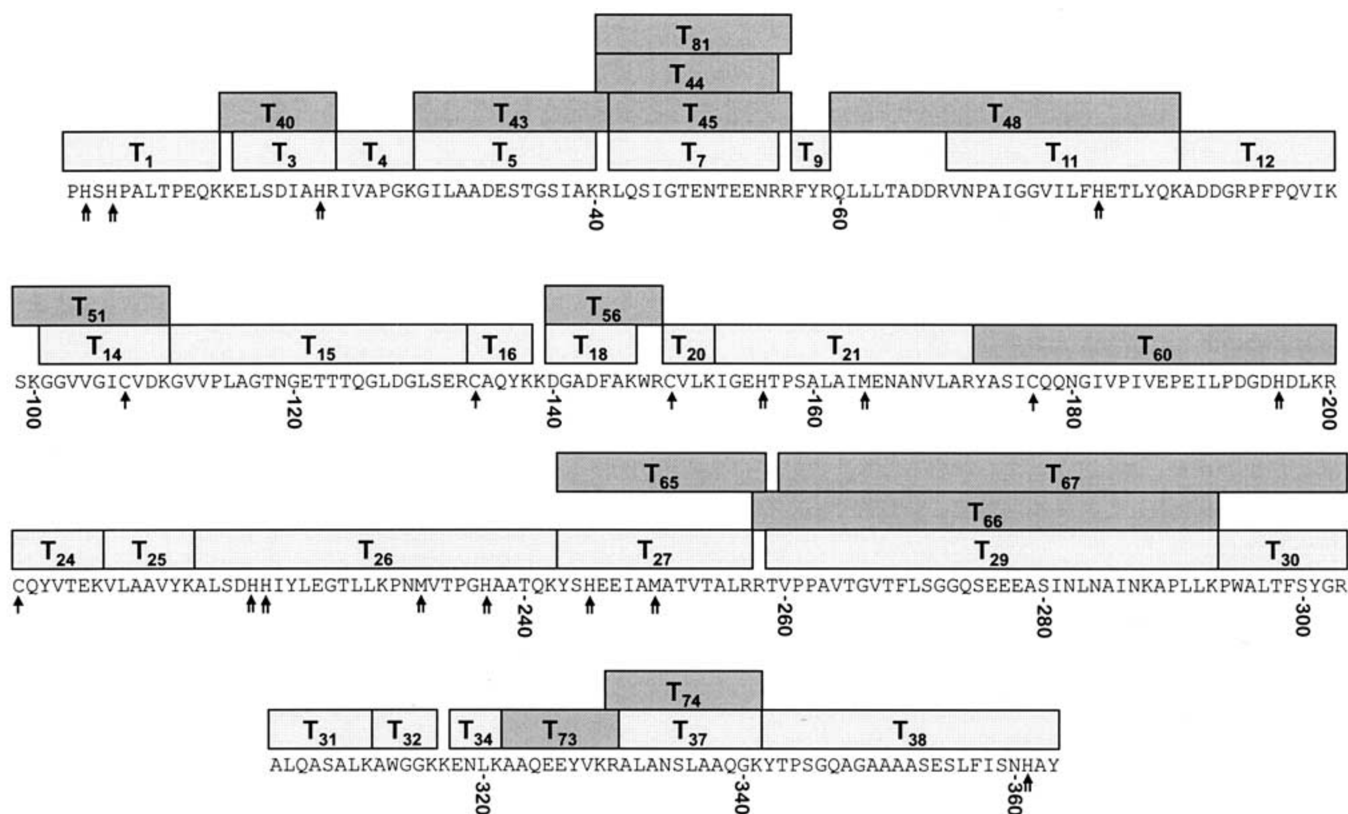

Fig. 3. Tryptic peptide map of tetK107C-EA aldolase. Assigned tryptic fragments are depicted by shaded boxes. Light-shaded boxes are completely digested peptides and dark-shaded boxes are incompletely digested peptides. Single arrows denote cysteine positions, and double arrows denote histidine or methionine positions. Amino acid is derived from the DNA sequence of the gtet expression plasmid with numbering as defined by Tolan et al. (1984). Nomenclature for tryptic peptides as defined in Figure 2 (Chowdhury et al. 1990).

Inc.) for mutagenesis were: geneU, acgaccgagcgcagcgagtcagtgag; 72U, gaaccccgccatcgggggcgtc; 72L, gacgcccccgatggcggggttc; 107U, gttgtgggcatctgcgtagacaagg; 107L, tgtctacgcagatgcccacaa cacc; 239U, cctggccatgccgccacccagaagtattct; 239L, gagaatacttc tgggtggcggcatggccagg; 289U, caacaaggccccccctgctgaagccgtg; 289L, tcagcagggggggccttgttgatggcgt; 338U, cgccgcccaagggaagta caccccgag; 338L, gtacttcccttgggcggcgaggctgttgg; geneL, gctactgc-cgccaggcaaactgttttatca. Mutagenic pairs of primers: 72U/72L, 239U/239L, 289U/289L, 338U/338L were used to substitute alanine for cysteine codons. Individual amplicons (geneU/72L, 72U/239L, 239U/289L, 289U/338L, 338U/geneU) from pPB14 (1.0 fmole) were gel purified, combined, and used for PCR with the flanking primer pair geneU/geneL, which anneals outside the coding region and *Eco*R I/*Hin*d III restriction sites. The flanking PCR product was phenol/chloroform extracted, ethanol precipitated, and *Eco*R I/*Hin*d III digested for insertion into pPB1. The resulting mutant, gtet, was used as the background for construction of tetK107C. Mutagenic primer pairs for tetK107C were geneU/107L and 107U/geneL. The clones were confirmed by DNA sequencing on an ABI 377 sequencer using the big dye terminator method (Applied Biosystems, Inc.).

Protein expression and purification

Six clones were cultured overnight and the clone exhibiting greatest expression by SDS-PAGE was used to inoculate 1L of 2xYT media and grown for 24 h at 37°C. The cells were pelleted, lysed by a French press, and the lysate cleared by centrifugation at 25,000*g* for 45 min. Protein was precipitated by adding $(NH_4)_2SO_4$ to 70% saturation at 4°C. Centrifugation at 25,000*g* for 15 min yielded a pellet that was resuspended in nitrogen-purged dialysis buffer (10 mM sodium phosphate, pH 6.7, 50 mM EDTA, 1 mM DTT, 25 mM PMSF, and 25 ng/mL pepstatin A). DNase I and RNase A were added at 5 mg/mL. Following dialysis, the protein was loaded on a (CM-Sepharose fast-flow column (Amersham Pharmacia Biotech, Inc.) in 0.5× dialysis buffer, washed with TGK buffer (50 mM Taps, 25 mM glycine, 25 mM KOH, pH 8.3, 0.1 mM DTT, 25 mM PMSF, and 25 ng/mL pepstatin A), and eluted with TGK buffer containing 0.5 mM Fru1,6P2. The eluate was precipitated in 70% $(NH_4)_2SO_4$. Aldolase (100–200 g/L culture) was 95% pure as determined by SDS-PAGE. Aliquots were removed and dialyzed in 1× dialysis buffer. Protein concentration was determined based on absorbance at 280 nm ($\varepsilon^{0.1\%} = 0.91$) (Baranowski and Niederland 1949).

Enzyme kinetics

Measurement of enzyme activity employed a coupled-enzyme assay (Racker 1952) at 30°C on a SpectroMAX 190 (Molecular Devices, Inc.). In a microtiter plate, reactions were composed of protein (50 μL of either 0.1 μM or 5 μM in subunits) and cocktail

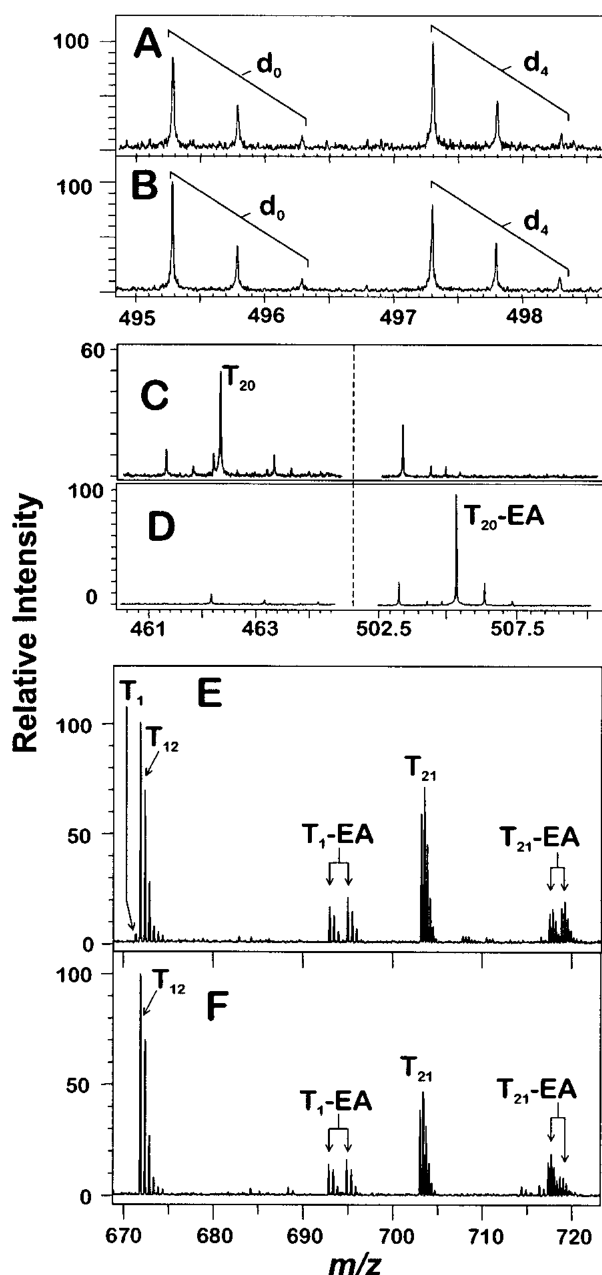

Fig. 4. Mass spectra of selected ions from tryptic digest of tetK107C–EA. (*A*) Double labeling of C107-containing T_{14} peptide under native conditions. The d_0 bracket denotes isotope peaks of T_{14} reacted with Br-CH_2-CH_2-NH_3^+ (50 mM) and d_4 bracket denotes reaction with Br-CD_2-CD_2-NH_3^+ (50 mM). (*B*) Material from (*A*) was denatured and reacted with d_0-BrEA (100 mM). (*C*) Two regions of *m/z* spectrum corresponding to positions of unmodified (*m/z* 462.29) and modified (*m/z* 505.33) T_{20} peptide under native conditions. (*D*) Sample treated as for (*C*) after subsequent labeling under denaturing conditions. (*E*) Peptides, T_1 and T_{21}, exhibiting aminoethylation at residues other than cysteine under native conditions. (*F*) As in (*E*), except under denaturing conditions.

buffer (150 µL of 100 mM TEA-Cl, pH 7.4, 20 mM Na-EDTA, 0.3 mM NADH, and 20 µg/mL GDH/TIM) followed by addition of substrate Fru1,6P2 (100 µL of 0.94 to 540 µM). Assays for specific activity toward Fru1P were done similarly with 20 mM Fru1P.

Synthesis of deuterated 2-bromo-ethylamine

Deuterated BrEA was synthesized from deuterated ethanolamine and HBr by modification of the method of Wystrach et al. (1955). In a hood, ethanol-1,1,2,2-d_4-amine (0.5 mg) was added dropwise to HBr (conc.) in a distillation flask and heated to boiling (105°C). The solution was refluxed at 135–140°C for 1 h, cooled for ~10 min, fitted to a vacuum distillation apparatus (~100 mTorr), and reheated until the solution volume was 0.1× starting. The solution was maintained at 105–110°C for 3–4 h until reflux stopped. Product was dissolved in H_2O. The yield was 70–80% as determined by TLC on dried silica gel plates pretreated with 0.5 M sodium phosphate solution (dibasic, pH 8.9). Samples were mobilized using 0.5 M dibasic Na_2HPO_4:acetone (1:19) and visualized with iodine vapor (BrEA = 0.54 rf, ethanolamine = 0.03 rf).

Protein aminoethylation

Aminoethylation was initiated by addition of BrEA (50 mM) into buffer (AMPSO, pH 9.5, or Tris-Cl, pH 8.9) (200 mM) followed

Table 3. *Observed double-labeled modifications of aldolase tryptic peptides*

Cysteine peptides	Calculated *m/z* (charge)	Native observed *m/z* (intensity)	Denatured observed *m/z* (intensity)
T_{14}-(d_0)-EA	495.28 (2)	495.29 (4.10)	495.29 (7.53)
T_{14}-(d_4)-EA	497.28 (2)	495.30 (4.68)	497.30 (6.65)
T_{51}-(d_0)-EA	602.85 (2)	602.86 (3.44)	602.86 (4.11)
T_{51}-(d_4)-EA	604.85 (2)	604.87 (3.95)	604.87 (4.04)
T_{16}-(d_0)-EA	655.35 (1)	n.o.[a]	655.34 (2.50)
T_{20}-(d_0)-EA	505.35 (1)	n.o.	505.33 (37.01)
T_{24}-(d_0)-EA	457.23 (2)	n.o.	457.24 (3.01)
T_{60}-(d_0)-EA	1055.22 (3)	n.o.	1055.25 (6.26)
Peptides containing residues other than cysteine			
T_1-(d_0)-EA	692.88 (2)	692.89 (6.35)	692.89 (5.67)
T_1-(d_4)-EA	694.88 (2)	694.90 (7.91)	694.91 (6.44)
T_3-(d_0)-EA	492.28 (2)	492.28 (1.10)	492.28 (3.19)
T_3-(d_4)-EA	494.28 (2)	494.29 (1.33)	494.29 (2.20)
T_{11}-(d_0)-EA	1021.57 (2)	n.o.	Conflict[b]
T_{11}-(d_4)-EA	1023.57 (2)	n.o.	Conflict[b]
T_{21}-(d_0)-EA	717.39 (3)	717.41 (4.69)	717.41 (5.87)
T_{21}-(d_4)-EA	718.72 (3)	718.74 (5.58)	718.74 (3.54)
T_{26}-(d_0)-EA	1029.55 (3)	1029.57 (1.18)	1029.58 (4.80)
T_{26}-(d_4)-EA	1030.88 (3)	1030.91 (1.21)	1030.90 (1.36)
T_{27}-(d_0)-EA	867.95 (2)	867.95 (<1)	867.98 (6.03)
T_{27}-(d_4)-EA	869.95 (2)	869.98 (<1)	869.97 (1.09)
T_{38}-(d_0)-EA	1143.05 (2)	n.o.	1143.09 (1.94)
T_{38}-(d_4)-EA	1145.05 (2)	n.o.	1145.10 (<1)
T_{40}-(d_0)-EA	556.32 (2)	n.o.	556.32 (1.32)
T_{48}-(d_0)-EA	1023.24 (3)	n.o.	1023.23 (1.59)
T_{65}-(d_0)-EA	946.00 (2)	n.o.	946.03 (<1)

[a] Not observed.
[b] Minor peak of T_{11}-EA overlapped with the major peak that corresponded to a peak consistent for T_{26} in which the Met is oxidized (T_{26}-Met-ox), which prevented definitive assignment.

Table 4. *Frequency and amount of aminoethylation*

Cysteine peptides	Probable sites	Freq.[a]	Ratio[b]
T_{51}	C107	1	0.86
T_{14}	C107	1	0.77
T_{16}	C134	1	nc[c]
T_{20}	C146	1	nc
T_{24}	C201	1	nc
T_{60}	C177, H196	3	nc
Peptides containing residues other than cysteine			
T_{1}	N-term, H2, H4	3	0.91
T_{3}	H20	1	nc
T_{11}	H80	0	nc
T_{21}	H156, M164	2	nc
T_{26}	H220, H221, H238, M232	4	nc
T_{27}	H245, M250	1	nc
T_{38}	H361	2	nc
T_{40}	H20	1	nc
T_{48}	H80	2	nc
T_{65}	H245, M250	1	nc

[a] Frequency of modification.
[b] Native d_0/d_4 ratio divided by denatured d_0/d_4 ratio.
[c] Not calculated. Either incomplete observation or d_4 peak not detected in either native or denatured spectra.

by addition of protein (50 μM). Reaction at 25°C was monitored by activity assays. For ESI-FT-MS of intact protein, reactions were terminated at 24 h by dialysis into 100 mM ammonium acetate at 4°C.

Double labeling

Two preparations of modified aldolase were generated, native and denatured. In the native preparation, a BrEA mixture resulting in 50 mM d_0-BrEA (Fluka, Inc.) and 50 mM d_4-BrEA was buffered at 200 mM Tris-Cl, pH 8.9. Reaction was initiated by addition of protein (100 μM), incubated for 22 h at 20°C, and terminated by dialysis at 4°C. For the denatured preparation, an aliquot of the protein treated as above was further treated by buffer exchange into 200 mM Tris-Cl pH 8.9, 4 M urea using a microcon-30 concentrator (Millipore, Inc.) and modified with d_0-BrEA (200 μM protein and 100 mM d_0-BrEA, 37°C, 14 h). This concentration of urea was sufficient to allow complete modification of buried cysteines (see Results). Both native and denatured samples were dialyzed twice against H_2O, and again against 100 mM ammonium acetate. Trypsin digestion (1:200 w/w) was at 37°C for 15 h.

Mass spectrometry

The FTMS was performed on a modified IonSpec system employing a 7T active-shielded superconducting electromagnet using a capacitively coupled closed cylindrical Penning trap. An electrospray ion source (Analytica, Inc.) was modified with a home-built nano-ESI interface using pulled glass capillary tips. Samples were analyzed with standard instrument operating parameters processed using varying degrees of truncation with at least one zerofill prior to Fourier transform with the Boston University Data Analysis (BUDA) software developed by the Boston University Mass Spectrometry Resource (P.B. O'Connor, personal communication). Deconvolutions were performed using the Fenn algorithm (Mann and Fenn 1989).

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