SUPPLEMENTAL INFORMATION

Mapping of the Cys-150 disulfide bond in IDH using 2-nitro-5-thiocyanobenzoate (NTCB):

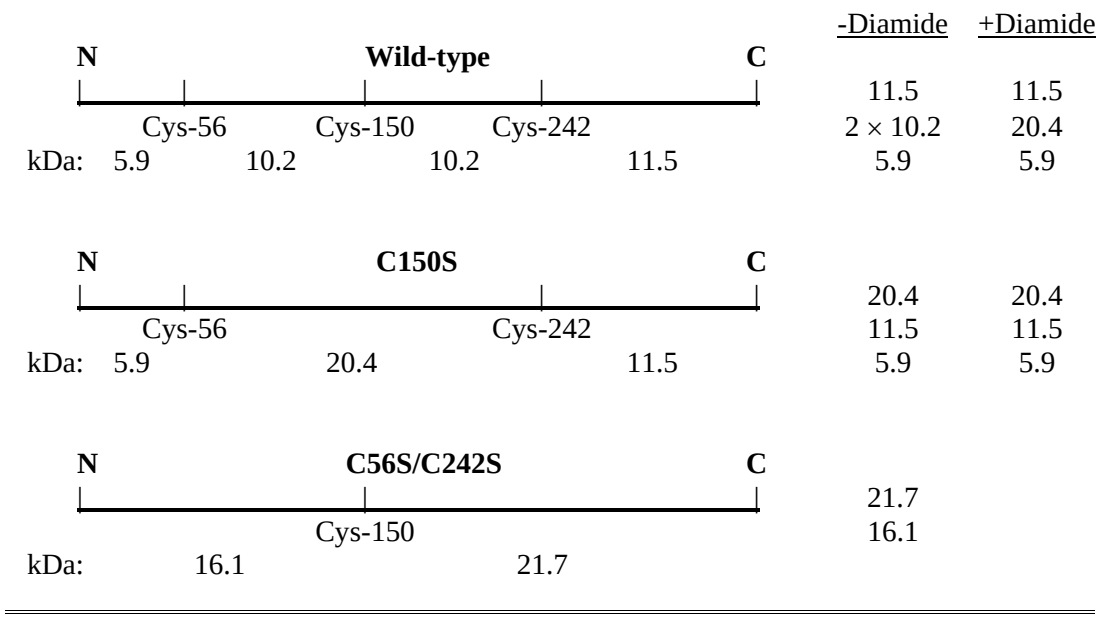
2-Nitro-5-thiocyanobenzoate (NTCB) is used for chemical cleavage of polypeptides at cysteine residues (*1*, *2*). At pH 8.0, this reagent interacts with free cysteine thiols to form an S-cyanocysteinyl side chain. The peptide chain is subsequently cleaved prior to the modified residue by cyclization induced by increasing the pH to 9.0. Since NTCB does not react with cysteine side chains involved in disulfide bonds, the reagent can be used to map positions of residues involved in formation of such bonds.

Treatment of affinity purified wild-type and mutant forms (C150S and C56S/C242S) of yeast IDH with NTCB was conducted essentially as previously described (*1*). 50 μg protein samples were incubated in 50 μl Tris/sodium acetate buffer (pH 8.0) containing 6 M urea at 37 ^{o}C for 30 min prior to addition either of 5 mM dithiothreitol to reduce any disulfide bonds or of 0.5 mM diamide to induce disulfide-bond formation. Incubations were continued for 30 min. NTCB was added to concentrations representing a 10-fold molar excess relative to potential sulfhydral groups in the protein or buffer in each sample, and incubations were continued for 30 min. To initiate protein cleavage by NTCB, the pH of the buffer was adjusted to 9.0 by addition of NaOH, and incubations were conducted at 37 ^{o}C for 18-20 h. Polypeptides were precipitated by addition of 1 ml 20% trichloroacetic acid and incubation at 4 ^{o}C for 16 h. Protein pellets were obtained by centrifugation for 15 min at 4 ^{o}C using a microcentrifuge

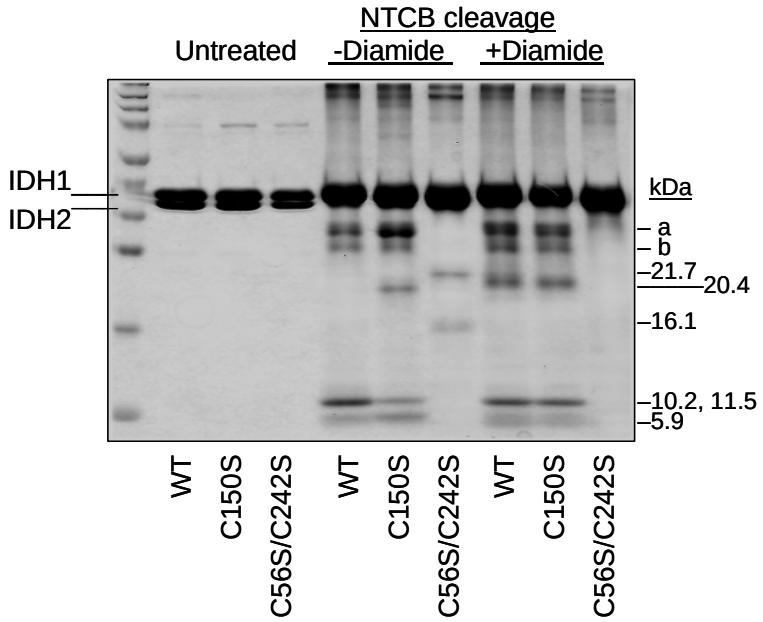
set at maximum speed. Pellets were washed twice by resuspending in 0.5 ml cold acetone (stored at -20 ^{o}C) and centrifugation prior to drying for 15 min at room temperature using a Speed Vac. Protein pellets were dissolved in 50 μl of a gel sample buffer containing 0.35 M β-mercaptoethanol for electrophoresis on 15% polyacrylamide SDS gels. The gels were stained with Coomassie blue.

Assuming that the Cys-150 disulfide bond in IDH2 is the only disulfide bond formed under conditions of treatment with diamide as described above, the expected patterns of diagnostic peptides for wild-type and mutant forms of IDH following cleavage with NTCB are illustrated in Figure 1A. Experimental results are presented in Figure 1B.

Figure 1: (A) Expected peptide cleavage patterns for the IDH2 subunit following treatment of reduced and oxidized forms of IDH enzymes with NTCB. With Cys-150 as the only residue competent for formation of a disulfide bond under described experimental conditions, NTCB cleavage is predicted to produce four diagnostic peptides from IDH2 for the reduced wild-type enzyme (-diamide), whereas three diagnostic peptides are expected for the diamide-oxidized enzyme (+diamide). Cleavage patterns for IDH2 in the C150S enzyme are expected to be similar under both reducing and oxidizing conditions. Two diagnostic peptides are expected for IDH2 in the reduced C56S/C242S enzyme, and none are expected for the oxidized enzyme.



(B) Experimental results following NTCB cleavage of wild-type and mutant forms (C150S and C56S/C242S) of IDH.



Experimental results for all enzymes indicated that the IDH1 subunit is not affected by NTCB treatment, as expected based on the absence of cysteine residues in this subunit. For the wild-type and C150S enzymes, the presence of bands designated **a** and **b** (with approximate sizes of 32 kDa and 26 kDa, respectively) is likely indicative of incomplete cleavage of IDH2 by NTCB as reported by other investigators (*2, 3*) and/or to the involvement of Cys-56 and Cys-242 residues in disulfide bonds in diamide-treated samples. This limits quantitative interpretation of results. For the wild-type enzyme, NTCB treatment of the reduced enzyme produced small bands with sizes appropriate for diagnostic fragments of ~10.2 kDa and 11.5 kDa, which are electrophoretically indistinguishable, and of ~5.9 kDa. In contrast, NTCB treatment of the diamide-oxidized wild-type enzyme produced the ~20.4 kDa band as predicted if Cys-150 residues are forming disulfide bonds.

NTCB cleavage patterns for the C150S enzyme were essentially identical with those of the diamide-oxidized wild-type enzyme and were largely unchanged by reducing or oxidizing conditions. These are the expected results for a mutant enzyme which cannot form Cys-150 disulfide bonds under these experimental conditions.

For the C56S/C242S enzyme, which contains Cys-150 as the sole cysteine residue, the NTCB cleavage patterns for the reduced enzyme were consistent with cleavage of IDH2 at Cys-150, producing bands of ~21.7 kDa and ~16.1 kDa. No cleavage of IDH2 was observed for the diamide-oxidized mutant enzyme, a result consistent with formation of the Cys-150 disulfide bond under these conditions.

References

1. Smith, B. J. (2003) Chemical cleavage of polypeptides, *Methods Mol. Biol. 211,* 63-82.

2. Stark, G. R. (1977) Cleavage at cysteine after cyanylation, *Methods Enzymol. 47,* 129-132.

3. Lu, H. S., and Gracy, R. W. (1981) Specific cleavage of glucosephosphate isomerases at cysteinyl residues using 2-nitro-5-thiocyanobenzoic acid: analyses of peptides eluted from polyacrylamide gels and localization of active site histidyl and lysyl residues, *Arch. Biochem. Biophys. 212*, 347-359.