Pergamon

PII: S0031-9422(97)00659-6

Phytochemistry, Vol. 47, No. 5, pp. 707–713, 1998
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Printed in Great Britain
0031-9422/98 $19.00+0.00

THE ENOLASES OF ICE PLANT AND *ARABIDOPSIS* CONTAIN A POTENTIAL DISULPHIDE AND ARE REDOX SENSITIVE

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(*Received in revised form* 1 *April* 1997)

Key Word Index—*Arabidopsis thaliana*; Brassicaceae; thale cress; *Mesembryanthemum crystallinum*; Aizoaceae; common ice plant; enolase; activity modulation; redox-sensitive cysteines.

Abstract—The simulated structures of the enolases of *Arabidopsis* and the common ice plant contain a pair of Cys residues in the correct orientation to form a disulphide bond. Formation of this disulphide might be expected to affect the positioning of several residues in the active site. The enzyme in crude extracts of these two plants is activated by oxidation. Apparently formation of the disulphide crosslink enhances catalysis. The enolases from tomato leaves, maize roots and castor bean embryos lack one of these Cys residues and are not redox sensitive. It seems possible that enolase is redox-regulated by a cytosolic thioredoxin system in a limited number of plant species including ice plant and *Arabidopsis*. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Although disulphides are common in extracellular proteins, in non-photosynthetic species intracellular disulphides are unusual and cysteine (Cys) residues positioned to form them are almost non-existent. In contrast, within chloroplasts redox regulation is an important regulatory mechanism and the modulated enzymes contain pairs of oxidizable cysteines. Recent experiments [1] suggest that the plant cytosol is also significantly more oxidizing than the cytosol of non-oxygenic species. We [2, 3] have shown that three cytosolic enzymes, namely barley lactate dehydrogenase, ice plant glyceraldehyde-3-P dehydrogenase and potato fructose bisphosphatase, are redox sensitive. There is experimental evidence [4, 5] to suggest that the redox sensitivity of the cytosolic fructose bisphosphatases is metabolically significant. It seemed possible that additional enzymes in the plant cytosol would contain Cys residues juxtaposed to form disulphides. Here we show that the enolases (EC 4.2.1.11) in the common ice plant and *Arabidopsis thaliana*, as modelled, contain a pair of Cys residues positioned to form a disulphide and are redox sensitive. The enolases in tomato, maize and castor bean, in which one of the Cys residues of the pair has been replaced by isoleucine, are redox insensitive under the same conditions.

RESULTS AND DISCUSSION

Enolase consists of a small N-terminal domain and a large C-terminal domain that is an $\alpha\beta$ barrel with the active site at the bottom [6]. There is an 'active site loop' (residues 35–45) which moves into place when substrate is bound [7, 8] and is thought to protect the reaction intermediate from solvent. (Numbering throughout is according to baker's yeast enolase, entry 5ENL in the Brookhaven Protein Data Bank, [9].) When we aligned the available sequences of the ice plant, *Arabidopsis*, maize (*Zea mays*), rice (*Oryza sativa*) and tomato (*Lycopersicon esculentum*) enolases (Figs. 1 and 2) and modelled the tertiary structure of each, we found a potential disulphide between Cys-313 and Cys-339 in the ice plant and *Arabidopsis* enzymes (Fig. 3). The αcarbon separation of residues 313 and 339 is 9.3 Å in Protein Data Bank entry 5ENL and the orientation would permit disulphide bond formation between two Cys residues. Cys-313 and -339 are located in the loops between αhelix-10 and βstrand-5 and αhelix-12 and βstrand-6. The disulphide would be accessible to thioredoxin. When lobster enolase (Protein Data Bank entry 1PDY, [10]) is used as the model enzyme these same residues appear to be capable of forming a disulphide bond (not shown).

Formation of the disulphide between Cys-313 and

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maize 1  MAVTITWVKARQIFDSRGNPTVEVDVGLSDGSYARGAVPSGASTGIYEALELRDGGSD.YLGKGVLKAVSNVNNIIGPAIVGKDPTEQVEID.NFMV
maize 2  MAATIQSVKARQIFDSRGNPTVEVDVFCSDGTFARAAVPSGASTGVYEALELRDGGSY.YLGKGVSKAVNNVNSVIGPALIGKDPTAQTEID.NFMV
  ice 1  .MVTIKCVKARQIYDSRGNPTVEADIHLDDGTYARAAVPSGASTGVYEALELRDGGKD.YMGKGVYKAVKNVNEIIGPALVGKDPTQQTAID.NFMV
  ice 2  .MVTIKCVKARQIYDSRGNPTVEADIHLDDGTYARAAVPSGASTGVYEALELRDGGKD.YMGKGVYKAVKNVNEIIGPALVGKDPTQQTAID.NFMV
 castor  MAITIVSVRARQIFDSRGNPTVEADIKLSDGHLARAAVPSGASTGIYEALELRDGGSD.YLGKGVSKAVENVNSIIGPALIGKDPTEQTALD.NFMV
  Arabi  .MATITVVKARQIFDSRGNPTVEVDIHTSNGIKVTAAVPSGASTGIYEALELRDGGSD.YLGKGVSKAVGNVNNIIGPALIGKDPTQQTAID.NFMV
  tom 2  .................................................................................................
  tom 1  .MATIKSIKARQIFDSRGNPTVEVDVHISNGVFARAAVPSGASTGIYEALELRDGGSD.YLGKGVSKAVNNVNSIIGPALVGKDPTDQTGLD.NFMV
  alder  .MAEITHIKARQIFDSRGNPTVEAEVTTANGVVSRAAVPSGASTGVYEALELRDGGSD.YLGKGVLKAVDNVNKIIGPALIGKDATEQTAIDIDFMF
   5ENL  ...AVSKVYARSVYDSRGNPTVEVELTTEKGVF.RSIVPSGASTGVHEALEMRDGDKSKWMGKGVLHAVKNVNDVIAPAFVKANIDVSDQKAVDDFL  93
               S9-------   S10-------   S11----                           H1----------------      H2------

maize 1  QQLDGTSNEWGWCKQKLGANAILAVSLAVCKAGAMVKKIPLYQHIANLAG.NKT.LVLPVPAFNVINGGSHAGNKLAMQEFMILPTGASSFKEAMKM
maize 2  QQLDGTKNEWGWCKQKLGANAILAVSLAVCKAGASIKRIPLYQHIANLAG.NKQ.LVLPVPAFNVINGGSHAGNKLAMQEFMILPTGAASFKEAMKM
  ice 1  QQLDGTVNEWGWCKQKLGANAILAVSLAVCKAGAQVKKIPLYQHIAEIAG.NKN.MVLPVPAFNVINGGSHAGNKLAMQEFMILPTGASSFKEAMKM
  ice 2  QQLDGTVNEWGWCKQKLGANAILAVSLAVCKAGAQVKKIPLYQHIAEIAG.NKN.MVLPVPAFNVINGGSHAGNKLAMQEFMILPTGASSFKEAMKM
 castor  QELDGTVNEWGWCKQKLGANAILAVSLALCKAGAHVKGIPLYKHIANLAG.NKN.LVLPVPAFNVINGGSHAGNKLAMQEFMILPVGASSFKEAMKM
  Arabi  HELDGTQNEWGWCKQKLGANAILAVSLAVCKAGAVVSGIPLYKHIANLAG.NPK.IVLPVPAFNVINGGSHAGNKLAMQEFMILPVGAASFKEAMKM
  tom 2  ................LGANAILAVSLAVCKAGAAVKKIPLYKHIANLAG.NKK.LVLPVPAFNVINGGSHAGNKLAMQEFMILPVGASSFKEAMKM
  tom 1  HQLDGTQNEWGWCKEKLGANAILAVSLAVCKAGAAVRNVPLYKHIADLAG.NKK.LVLPVPAFNVINGGSHAGNKLAMQEFMILPVGAANFKEAMKM
  alder  QQLDGTVNEWGWCKQKLGANAILAVSLAVCKAGASVKKIPLYKHIANLAG.NPK.LVLPVPAFNVINGGSHAGNKLAMQEFMILPVGASSFKEAMKM
   5ENL  ISLDGTAN.....KSKLGANAILGVSLAASRAAAAEKNVPLYKHLADLSKSKTSPYVLPVPFLNVLNGGSHAGGALALQEFMIAPTGAKTFAEALRI 185
         ----              H3----------------   H4------       S1----------             S2----   H5-----

          187
           |
maize 1  GVEVYHNLKSIIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKAAIEKAGYTGKVVIGMDVAASEFFGEKDKTYDLNFKEENNDGSNKISGDSLKD
maize 2  GVEVYHHLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYSDKDQTYDLNFKEENNDGSQKISGDSLKN
  ice 1  GSEVYHNLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYK.EDKSYDLNFKEENNDGSQRISGEALKD
  ice 2  GSEVYHNLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYK.EDKSYDLNFKEENNDGSQRISGEALKD
 castor  GAEVYHHLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIAKAGYTGKVVIGMDVAASEFYG.SDKTYDLNFKEENNDGSQKISGEALKD
  Arabi  GVEVYHHLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYS.EDKTYDLNFKEENNNGSQKISGDALKD
  tom 2  GCEVYHHLKAVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYG.KDKTYDLNFKEENNNGSQKISGDQLKD
  tom 1  GCEVYHHLKAVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYTGKVVIGMDVAASEFYG.KDKSYDLNFKEESNDGSQKISGDQLKD
  alder  GVEVYHHLKAVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIAKAGY...VVIGMDVAASEFYEKDKDI..TNFKEENNDGSQKISADQLKD
   5ENL  GSEVYHNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIKAAGHDGKVKIGLDCASSEFFKDG..KYDLDFKNPNSDKSKWLTGPQLAD 280
         ---------------  H6--              H7------------      S3----  H8-                        H9----

                                        313                         339
                                         |                           |
maize 1  LYKSFVSEYPIESIEDPFDQDDWSTYAKLTDEIGQKVQIVGDDLLVTNPTRVAKAINEKTCNALLLKVNQIGSVTESIEAVRMSKRAGWGVMASHRS
maize 2  VYKSFVSEYPIVSIEDPFDQDDWVHYAKMTEEIGEQVQIVGDDLLVTNPTRVAKAIKEKSCNALLLKVNQIGSVTESIEAVKMSKRAGWGVMTSHRS
  ice 1  LYKSFVAEYPIVSIEDPFDQDDWEHYAKMTAECGEKVQIVGDDLLVTNPKRVKKAIDENPCNALLLKVNQIGSVTESIEAVKMSKKAGWGVMASHRS
  ice 2  LYKSFVAEYPIVSIEDPFDQDDWEHYAKMTAECGEKVQIVGDDLLVTNPKRVKKAIDENSCNALLLKVNQIGSVTESIEAVKMSKKAGWGVMASHRS
 castor  LYKSFASEYPIVSIEDPFDQDDWEHYSKLTSEIGEKVQIVGDDLLVTNPKRVEKAIQEKACNALLLKVNQIGSVTESIEAVRMSKRAGWGVMASHRS
  Arabi  LYKSFVAEYPIVSIEDPFDQDDWEHYAKMTTECGTEVQIVGDDLLVTNPKRVAKAIAEKSCNALLLKVNQIGSVTESIEAVKMSKKAGWGVMTSHRS
  tom 2  LYKSFVSEYPIVSIEDPFDQDDWETYAKLTAEIGQKVQIVGDDLLVTNPKRVAKAISEKTCNALLLKVNQIGSVTESIEAVKMSKQAGWGVMTSHRS
  tom 1  LYKSFVSEYPIVSIEDPFDQDDWETYAKLTAEIGEQVQIVGDDLLVTNPKRVAKAIAEKTCNALLLKVNQIGSVTESIEAVKMSKKAGWGVMTSHRS
  alder  LYKSFVDEYPIVSIEDPFDQDDWEHYSKLTAEIGEKVQIVGDDLLVTNPKRVEKAIKEKACNALLLKVNQIGSVTESIEAVKMSKRAGWGVMA.HRS
   5ENL  LYHSLMKRYPIVSIEDPFAEDDWEAWSHFFKTAG..IQIVADDLTVTNPKRIATAIEKKAADALLLKVNQIGTLSESIKAAQDSFAAGWGVMVSHRS 375
         --------   S4-       H10------       S5--H11-   H12------    S6----H13-  H14----------   S7----

                                 401
                                  |
maize 1  GETEDTFIADLSVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGDAAVYAGAKFRAPVEPY
maize 2  GETEDTFIADLAVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGAIAVYAGAKFRAPVEPY
  ice 1  GETEDTFIADLSVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGDKAVYAGANFRRPVEPY
  ice 2  GETEDTFIADLSVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGDKAVYAGANFRRPVEPY
 castor  GETEDTFIADLSVGLATGQIKTGAPCRSERLAKYNQLLRIEEELGAEAVYAGAKFRTPVEPY
  Arabi  GETEDTFIADLAVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGSEAIYAGVNFRKPVEPY
  tom 2  GETEDTFIADLAVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGSDAVYAGAS.......
  tom 1  GETEDTFIADLAVGLSTGQIKTGAPCRSERLAKYNQLLRIEEELGSEAVYAGASFRKPVEPY
  alder  GETEDTFIADLSVGLATGQIKTGAPCRSERLAKYNQLLRIEEELGSEAVYAGANFRTPVEPY
   5ENL  GETEDTFIADLVVGLRTGQIKTGAPARSERLAKLNQLLRIEEELGDNAVFAGENFHHGDKL. 436
               H15------   S8-      H16--------------
```

Fig. 1. Sequence alignment of the enolases from maize (maize 1, Genbank X55981; maize 2, Genbank U17973), ice plant (ice 1, Genbank S79242; ice 2, Genbank U09194), castor bean (Genbank Z28386), *Arabidopsis* (Arabi, Genbank X58107), tomato (tom 2, Genbank X58109; tom 1, Genbank X58108 as Protein Information Resource entry JQ1186), alder (Genbank X92377) and yeast (Protein Data Bank 5ENL). Cys residues are in bold. Secondary structure from the Protein Data Bank file for the yeast enzyme is given with '*S*' indicating a βstrand and '*H*' indicating an αhelix. Strands 9, 10, and 11 are numbered according to Lebioda *et al.* [31]. Not shown are 48 additional enolase sequences available on January 1, 1996 in the GenBank, Swiss-Protein and Protein Data Bank data bases which were included in the alignment. The identities after alignment with 5ENL are Genbank X55981, 59%; U17973, 58%; S79242, 60%; U09194, 60%; Z28386, 59%; X58107, 59%; X58108, 60%; and X92377, 58%.

-339 might be expected to affect Asp-320 which binds Mg I, the high-affinity Mg^{2+} [8, 9, 11], and/or Lys-345 which functions as the base in the ionization of 2-P-glycerate [12, 13]. Asp-320 is located immediately after the C terminus of βstrand-5. The position and orientation of its side chain might shift as a result of movement of Cys-313 in the loop at the other end of βstrand-5. Lys-345 is located at the C terminus of βstrand-6, which might be expected to move if Cys-339, adjacent to the N terminus of βstrand-6, moves when the disulphide is formed (see Figs 3 and 4). The precise effects will depend on the relative amount of movement of each of the two Cys residues involved in the formation of the disulphide. A change in the positioning of Mg I might also affect Arg-374, which is repelled by the positive electrostatic field surrounding Mg I. Arg-374 binds the phosphate of the carbon substrate [6]. Cys-313 and -339 are not part of the

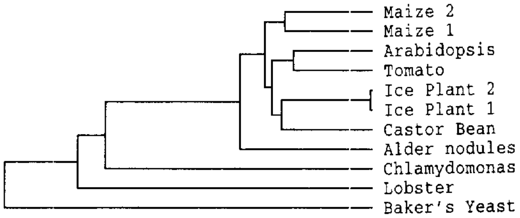

Fig. 2. Dendrogram of the sequences of enolases from maize, ice plant, castor bean, alder, tomato, *Chlamydomonas reinhardtii*, lobster, baker's yeast and *Arabidopsis*. Only the complete tomato enolase sequence (Genbank X58108) was used. *Chlamydomonas reinhardtii* sequence from Genbank X66412. For remaining Genbank numbers see Fig. 1 legend.

active site loop or the other two loops which move [7] when substrate is bound. There were no Cys residues close enough to one another to suggest the possibility of inter-domain disulphide bond formation in any of the plant enolases included in this study.

It is interesting that the sole difference between the two enolase isoforms in ice plant is at residue 338, which is Ser in one and Pro in the other. A Pro between a redox-sensitive Cys residue and a structural element (here *β*strand-6) might be expected to decrease the rotational mobility of the Cys thereby affecting the redox potential of the reaction and disulphide bond formation.

The available sequence information indicates that the enolases in tomato leaves, castor bean (*Ricinus communis*) cotyledons and maize roots contain Cys-339, but not Cys-313. Although Cys-339 is found in some non-plant enolases, Cys-313 is not found in any other enolase sequences.

When we tested the effect of the reductant DTT and the oxidant diamide [14] on enolase activity in crude ice plant extracts we found that the enzyme was slightly inhibited by DTT treatment and was activated about 2-fold by diamide treatment. Apparently diamide is oxidizing two Cys residues to cysteine and the activity of the enzyme increases as a result of the formation of the disulphide. The fact that DTT has only a slight inhibitory effect on the activity of the enzyme indicates that a disulphide bond is not a requisite for catalysis.

The enzyme in *Arabidopsis* was activated when crude extracts were treated with either DTT or diamide. It is possible that the 30 or 40% increase in activity when *Arabidopsis* extracts are treated with DTT is the result of cleavage of a mixed disulphide between enolase and some other thiol compound. It is also possible that the activity assayed in these crude extracts represents two or more isoforms (see below). Whatever the explanation, it is clear that, as predicted, there are redox-sensitive enolases in both *Arabidopsis* and the ice plant.

DTT and diamide had little or no effect on the activity of the enzyme in extracts of tomato leaves, castor bean cotyledons, or maize roots (Table 1). Clearly diamide treatment increases the activity of enolases that contain Cys-313 and Cys-339 and does not affect enzymes which contain only Cys-339. Apparently, formation of a cross-link between these two residues positively affects the activity of the enzyme. Because DTT is not an inhibitor of activity

Fig. 3. Predicted tertiary structure of ice plant enolase. The corresponding residues on the *Saccharomyces cerevisiae* enolase are replaced by the ice plant Cys residues (shown as spheres). The distance between the αcarbons of Cys residues 313 and 339 is 9 Å. Active site residues are shown in stick form and Mg I as a grey sphere. Numbering is according to the reference enzyme. We used coordinates (Entry 5ENL) [6] obtained from the Protein Data Bank at Brookhaven National Laboratory [29, 30]. Active site residues are according to [6, 9, 12]. This figure was generated using Molscript [32].

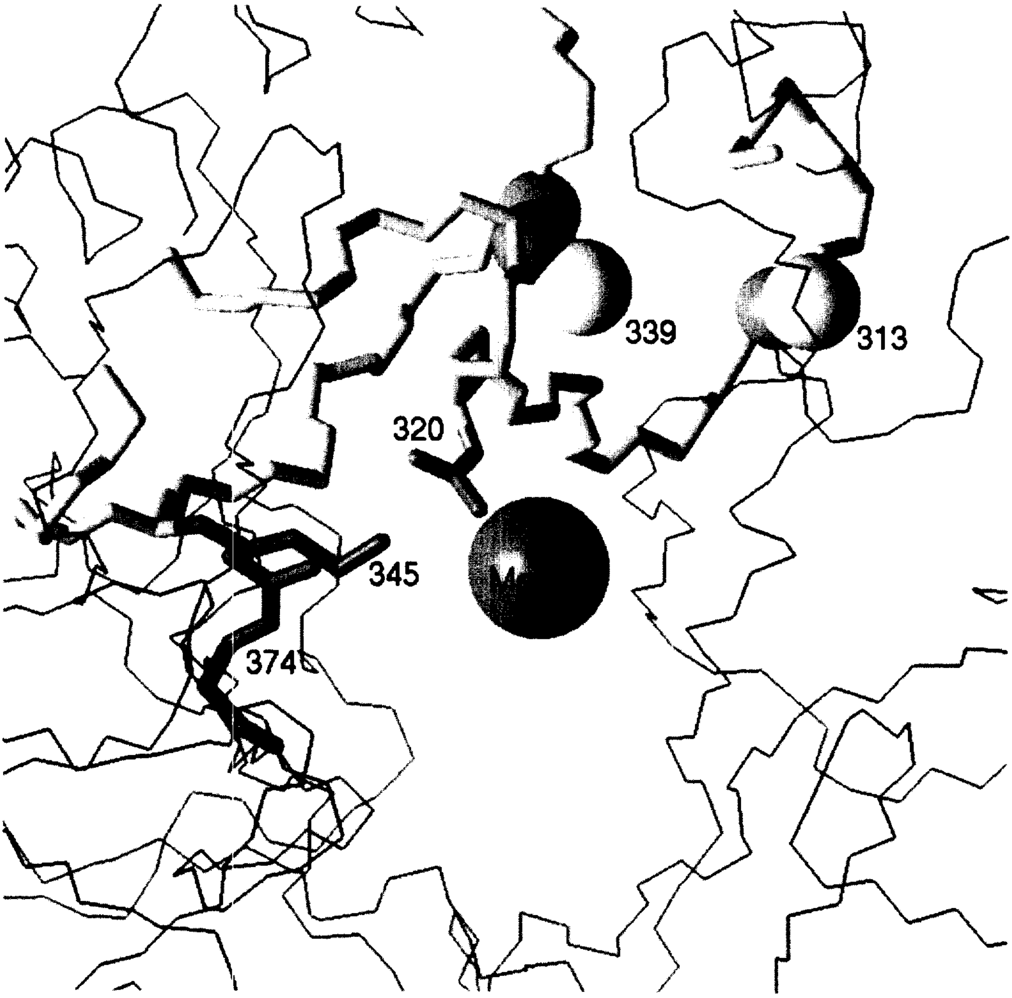

Fig. 4. Close-up of the active site region showing Cys-313 and Cys-339, the active site residues Asp-320, Lys-345 and Arg-374, and Mg I. Formation of a disulphide bond between the two Cys residues might be expected to affect the positioning of some or all of the active site residues. The diagram was made with INSIGHT II (Biosym Technologies, San Diego CA) on a Silicon Graphics work station.

Table 1. Effect of treatment with DTT and diamide on enolase activity in crude extracts

Plant source	Activity (nmol min^{-1} mg^{-1} protein)	Effect of DTT	diamide	Potential disulphide
		(% of control)		
Ice plant	146	90	211	313–339
Leaf	140	84	189	
Arabidopsis	91	137	150	313–339
Whole plant	77	130	150	
Rice	616	109	101	
Coleoptile	376	112	106	
Castor bean	212	110	103	
Cotyledon	254	92	101	
Tomato	66	102	95	187–401*
Leaf	51	95	90	
Maize	1010	87	100	
Root	757	81	94	

Values are means of triplicate assays. Each set of values represents an independent experiment.

* Not expected to be accessible to intracellular oxidants or reductants (see text).

in *Arabidopsis* and inhibits ice plant activity only very slightly, it seems unlikely that this oxidative enhancement was occurring in this tissue at the time these extracts were prepared.

There are several enolase genes in rice, tobacco, and tomato [15], and hence multiple forms might be expected in each of these plants. There is biochemical evidence for multiple forms of enolase in maize [16], and in castor bean and other oil seed plants there are plastid and non-plastid isoforms of the enzyme [17, 18]. In ice plant there appear to be two isozymes, differing only at residue 338 [19]. It is therefore unlikely that there is another isoform which is susceptible to oxidation and reduction and might account for the results found here with this plant. However, although Van der Straeten *et al.* [15] found evidence for a single gene in *Arabidopsis* in southern blot experiments, Newman *et al.* [20] found additional fragments corresponding in sequence to enolase DNA when they sequenced 1500 anonymous *Arabidopsis* cDNA clones. It is therefore possible that the results found with *Arabidopsis* represent two or more activities. Apparently the ice plant and *Arabidopsis* enolases contain redox-sensitive Cys residues. It is reasonable to assume that the oxidizable Cys residues are Cys-313 and Cys-339, although we cannot rule out the possibility that another set of Cys residues is responsible for the redox sensitivity of part of the enolase activity in *Arabidopsis*.

The tomato enolases for which there is sequence information (Genbank X58108 and X58109) contain another pair of Cys residues (Cys-187 and -401) that appear to be close enough (8.8 Å αcarbon separation) to form a disulphide bond. These two Cys residues are buried at the interface between the two subunits and would not be accessible to a small peptide oxidant or reductant. The enzyme in crude tomato leaf extracts was not affected by treatment with DTT or diamide (Table 1). Apparently then this disulphide does not form, or, if it does form, the activity of the enzyme is not affected. There were no other Cys residues positioned to form disulphide bonds in the tomato isozymes, and no Cys residues close enough to one another to form disulphides in the rice, castor bean or maize root enolases.

Although enolase activity increases in ice plant leaves during Crassulacean acid metabolism induction [19, 21] and transcript levels increase, enolase protein levels do not [19]. Therefore, there must be some type of post-translational modification of the activity of the enzyme. It seemed possible that the redox state of the enzyme might be affected by stress, but when we subjected ice plants to 24 hr of hypoxia, drought, or cold stress, or 72 hr of salt stress, we found no significant differences in the relative effects of DTT and diamide treatment (data not shown). There is then apparently no remarkable difference in the oxidation state of the enzyme in the leaf after these treatments. We note that anaerobic treatment leads to an increase in enolase activity without any increase in transcript level in maize [16, 22]. Because there appear to be no potential redox sensitive residues in the maize enolases some form of activity modulation which is not related to oxidation and reduction of these Cys residues must occur in maize and might then be expected to occur in other plants. Apparently this modulation does not involve phosphorylation: Mujer *et al.* [23] have shown that the enolase of *Echinochloa phyllopogon* is phosphorylated, but no effect of phosphorylation on activity.

If enolase is redox regulated in ice plant and *Arabidopsis*, modulation probably involves a cytosolic thioredoxin. It has been suggested that the reductively activated alternative oxidase in plant mitochondria is activated by reduced thioredoxin [24]. In transgenic plants with reduced levels of alternative oxidase activity there is an increase in the products of fermentative metabolism. Reductive activation of alternative oxidase or reductive inactivation of enolase would be expected to accomplish a decrease in the flow of carbon into fermentative end products. It seems possible that the oxidative activation of enolase observed here occurs *in vivo* as well as *in vitro* and that redox regulation of enolase and the alternative oxidase work together to protect plants from production of the undesirable products of anaerobic metabolism. It is also possible that the disulphide simply stabilizes the protein and has no regulatory role.

The *Arabidopsis* and ice plant enolases now become further examples of redox-sensitive cytosolic enzymes in higher plants. The others are the cytosolic fructose bisphosphatases [3–25], the unusual NAD-linked glyceraldehyde-3-P dehydrogenases of ice plant and salt plant [3], and barley lactate dehydrogenase [2]. In addition, the mitochondrial citrate synthases of at least three angiosperms are reductively activated [26]. To our knowledge this is the first example of an $\alpha\beta$-barrel structure enzyme being activated by formation of a disulphide bond.

EXPERIMENTAL

Plant material. Ice plant (*Mesembryanthemum crystallinum* L.) seed was provided by Hans J. Bohnert, University of Arizona; maize (*Zea mays* L. strain B73Ht) seed by Martin M. Sachs, University of Illinois, Urbana; rice (*Oryza sativa* L. strain S-201) seed by Jim Hill and Tim Kesselring, University of California, Davis, and *Arabidopsis thaliana* (ecotype Columbia) seed by Joseph J. Kieber, this department. Tomato (*Lycopersicon esculentum* L. cv Rutgers) seed was obtained from Carolina Biological Supply, Burlington NC. Castor bean (*Ricinus communis* L. commercial variety 'Tall Green Leaf') seed was from Lake Valley Seed, Boulder CO. Plants were grown in Sunshine Number 5 Mix (Sungrow Horticultural Inc. Canada) under natural light in a greenhouse.

Preparation of extracts. Ice plant and tomato extracts were prepared from the true leaves of young (1–1.5 months from planting) seedlings. *Arabidopsis* extracts were prepared from the entire seedling 1–2 months after planting. The cotyledons of castor bean plants 20 and 22 days after planting were used for extract preparation. Coleoptiles and a small amount of leaf tissue were used for preparation of rice tissue extracts 11–13 days after planting. Only the roots of the maize plants were used and $MgCl_2$ (10 mM) was included in the extraction buffer. The seedlings were 12 days old.

Plant material was washed in cold deionized water, rinsed in 50 mM pH 7.4 Tris–HCl buffer, and ground in a tissue homogenizer in about 5 ml of the buffer at 0–4°C. The brei was centrifuged (27 000 × g, 15 min) and the supernatant was decanted, made 50 mM in dithiothreitol (DTT) or 5 mM in diazinedicarboxylic acid bis(*N*,*N*-dimethylamide) (diamide) and allowed to stand for 30 min on ice.

Enzyme assay and protein estimation. Enolase (EC 4.2.1.11) activity was determined by a slight modification of the method of Westhead and Malmström [27]. The assay mixture was 50 mM Tris-HCl, pH 7.4, 1.2 mM 2-P-glycerate and 10 mM $MgCl_2$. EDTA was omitted. The assay temperature was 26°C. Protein was estimated by the method of Bradford [28] with bovine serum albumin as standard.

Modelling. The simulated structures of the plant enolases were generated by homology modelling from the structure of baker's yeast enolase (Protein Data Bank entry 5ENL). A multiple sequence alignment of the 59 enolase sequences available in the Protein Data Bank, GenBank, Swiss Protein and Protein Information Resource data bases was generated, using PileUp in the Wisconsin Sequence Analysis Package, Version 8 (Genetic Computer Group, Inc., Madison WI). Coordinates were obtained from the Protein Data Bank at Brookhaven National Laboratory [29, 30]. The tertiary structure diagrams were displayed and made with INSIGHT II (Biosym Technologies, San Diego CA) on a Silicon Graphics work station. No energy minimization procedures were used and no manual adjustments were made.

Construction of dendrogram. The dendrogram of the alignment was constructed with the PILEUP program in the Wisconsin Sequence Analysis Package, Version 8, Genetics Computer Group, Inc. The default settings were used.

Acknowledgements—This work was supported by US National Science Foundation (Grant MCB-9513498) and by US Department of Energy, Office of Health and Environmental Research, under contract W-31-109-ENG-38. We thank Hans J. Bohnert, University of Arizona for providing the ice plant seeds; Marty Sachs, University of Illinois-Urbana for providing maize seeds; Jim Hill and Tim Kesselring, University of California-Davis for providing rice seeds; Joe Kieber, this department, for providing *Arabidopsis* seeds; Syed S. Lateef for assistance with these experiments, and Larry Sykora and staff at the UIC Greenhouse for cultivating the plants.

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