

## 502. $\beta$ -Aspartyl dipeptidase

### Databanks

Peptidase classification: clan MX, family M38, MEROPS ID: M38.001

NC-IUBMB enzyme classification: none

Databank codes:

Species	SwissProt	PIR	EMBL (cDNA)	EMBL (genomic)
<i>Escherichia coli</i>	P39377	B55889	U15029	U14003: chromosome 92.8 to 00.1'

### Name and History

Isoaspartyl residues arise by the spontaneous post-translational alteration of aspartyl and asparaginyl residues in proteins (see Figure 502.1). This abnormal residue forms the peptide bond linkage through its side chain  $\beta$ -carboxyl group rather than its  $\alpha$ -carboxyl group. Several early studies using isoaspartyl-containing peptides showed that the isoaspartyl  $\beta$ -carboxyl bond was generally resistant to proteolysis. For example, extracts of yeast and mammalian cells that could hydrolyze the dipeptide aspartyl-glycine did not hydrolyze the corresponding isoaspartyl-glycine dipeptide (Grassmann & Schneider, 1934). Rat liver or kidney extracts demonstrated no hydrolytic activity towards an isoaspartyl-alanine dipeptide (Greenstein & Price, 1949). Finally, leucyl aminopeptidase (Chapter 473) was shown to hydrolyze the aspartyl-containing angiotensin II (Asp-Arg-Val-Tyr-Val-Ala-His-Pro-Phe) but not the corresponding isoaspartyl form (Riniker & Schwyzer, 1964). The first evidence for hydrolysis of a  $\beta$ -carboxyl linkage was the cleavage of an isoaspartyl-histidine dipeptide by a partially purified preparation of pig kidney carnosinase ( $\beta$ -alanyl-histidine hydrolase; Hanson & Smith, 1949).

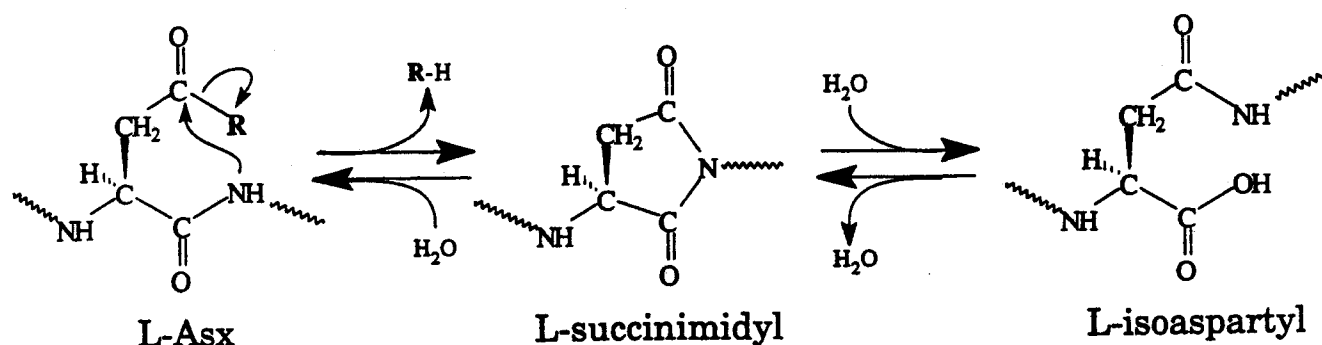
Consistent with the general resistance of isoaspartyl  $\beta$ -linkages to proteolysis, a number of isoaspartyl-containing peptides were found in normal human urine (Buchanan *et al.*,

1962). These peptides arise from endogenous metabolism as well as from dietary sources (Dorer *et al.*, 1966). The finding that the levels of various isoaspartyl peptides did not vary in the same proportions with food consumption (Dorer *et al.*, 1966) suggested the presence of a proteolytic mechanism that minimized the accumulation of isoaspartyl peptides. Interestingly, when rats were given radiolabeled isoaspartyl di- and tripeptides by stomach tube, only a small portion of the radioactivity was rapidly excreted, suggesting that these peptides could be metabolized by one or more enzymes that cleaved the isoaspartyl linkage (Dorer *et al.*, 1968). This rat enzyme is classified as EC 3.4.19.5 and its mechanistic class has never been determined. It is discussed separately in Chapter 551. An enzyme from *Escherichia coli* was partially purified by Haley (1968) and has been more recently purified to homogeneity (Gary & Clarke, 1995). Unlike the mammalian enzyme, this bacterial enzyme does not hydrolyze tripeptides and is therefore called  $\beta$ -aspartyl dipeptidase or isoaspartyl dipeptidase.

### Activity and Specificity

Isoaspartyl peptidase activity was first characterized in rat liver extracts (Dorer *et al.*, 1968) (Chapter 551). This activity, assayed by the hydrolysis of an L-isoaspartyl-glycine





**Figure 502.1** Aspartyl ( $R = -OH$ ) and asparaginyl ( $R = -NH_2$ ) residues can undergo a spontaneous intramolecular cyclization reaction in which the peptide-backbone nitrogen atom of the following residue attacks the carbonyl of the Asx side chain to form an L-succinimidyl residue (*center*). Upon hydrolysis, this cyclic product opens to give either the L-aspartyl residue or an L-isoaspartyl residue that now has the peptide bond connecting through the side-chain  $\beta$ -carboxyl group, resulting in a kink in the peptide backbone.

**Table 502.1** Dipeptidase activity

Substrate	% of maximal activity		
	Partially purified rat liver preparation <sup>a</sup>	Partially purified <i>E. coli</i> preparation <sup>b</sup>	Homogeneous <i>E. coli</i> enzyme <sup>c</sup>
$\beta$ -Asp-Gly	100	0	8
$\beta$ -Asp-Met	82	68	
$\beta$ -Asp-Leu	65	100	100
$\beta$ -Asp-Ser	56	82	
$\beta$ -Asp-Ala	51	33	
$\beta$ -Asp-Ile	37	19	
$\beta$ -Asp-Thr	29	18	
$\beta$ -Asp-Val	2	56	
$\beta$ -Asp-Gln		48	
$\beta$ -Asp-Phe		38	
$\beta$ -Asp-Asn		10	
$\beta$ -Asp-His		0	
$\beta$ -Asp-Gly-Gly	95	0	
$\beta$ -Asp-Gly-Ala	55	0	
$\beta$ -Asp-Gly-Val	13		
$\beta$ -Asp-Leu-Gly		0	
Asp-Gly	3		
Asp-Leu	9	0	13
$\gamma$ -Glu-Leu	0	0	0
$\gamma$ -Glu-Gly			0
$\gamma$ -Glu-His			0
$\gamma$ -Glu-Cys			0

<sup>a</sup>Dorer *et al.* (1968); <sup>b</sup>Haley (1968); <sup>c</sup>Gary & Clarke (1995).

dipeptide, was purified approximately 15-fold over crude cytosol and was maximally active in phosphate buffer at pH values between 7.5 and 8.0, falling off sharply below 6 and above 9 (Dorer *et al.*, 1968).

An analogous isoaspartyl dipeptidase activity was found shortly thereafter in the bacterium *Escherichia coli* (Haley, 1968). This activity was initially characterized using a preparation that was enriched 110-fold over crude cytosol (Haley, 1968) and has more recently been purified to homogeneity (Gary & Clarke, 1995). The pH activity profile of this

bacterial preparation is similar to that of the originally characterized rat enzyme (Haley, 1968). The *E. coli* enzyme, however, has little or no activity towards the isoaspartyl-glycine dipeptide (Haley, 1968; Gary & Clarke, 1995) (see Table 502.1). Instead, maximal activity is observed with an isoaspartyl-leucine dipeptide substrate which has a  $K_m$  of 0.81 mM (Haley, 1968). As with the mammalian enzyme, normal aspartyl and  $\gamma$ -glutamyl dipeptides are either not substrates or are much poorer substrates (Table 502.1) (Haley, 1968; Gary & Clarke, 1995). Interestingly, the *E. coli* enzyme

is unable to utilize isoaspartyl tripeptides as substrates (Haley, 1968). Another distinguishing feature of the *E. coli* enzyme activity is its sensitivity to  $Mn^{2+}$ ,  $Co^{2+}$  and  $Zn^{2+}$ , which inhibit the enzyme 42, 90 and 100%, respectively (Haley, 1968). On the other hand, the bacterial enzyme activity is unaffected by iodoacetamide (5 mM), *o*-iodosobenzoate (1 mM), and ammonium persulfate (0.4 mM), but is inhibited 17% by 2 mM *p*-hydroxymercuribenzoate (Haley, 1968).

For comparison, the partially purified rat liver enzyme was able to hydrolyze a number of isoaspartyl dipeptides; isoaspartyl-glycine was the best substrate tested (Table 502.1). In addition to hydrolyzing these dipeptides, the enzyme preparation also cleaved the N-terminal isoaspartyl peptide bond present in several tripeptides (Dorer *et al.*, 1968). The activity of this peptidase seems to be specific for isoaspartyl residues, because normal aspartyl- and isoglutamyl ( $\gamma$ -glutamyl)-containing dipeptides were not substrates (Dorer *et al.*, 1968). The rat liver isoaspartyl peptidase was neither greatly inhibited nor strongly activated by various divalent metal ions ( $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ca^{2+}$  and  $Co^{2+}$ ) at a concentration of 1 mM, the greatest effect being a 46% inhibition by 1 mM  $Zn^{2+}$  (Dorer *et al.*, 1968). Sodium or potassium ions were required for maximal activity (Dorer *et al.*, 1968). Activity was unaffected by sodium EDTA, 2-ME and iodoacetamide at 1 mM concentrations; however, 1 mM *p*-hydroxymercuribenzoate inhibited the activity by 70% (Dorer *et al.*, 1968).

On the basis of these differences, it seems best to retain two separate entries for the mammalian and bacterial peptidases. The mammalian enzyme cannot be definitively assigned to a class and so is treated with the unclassified peptidases in this *Handbook* (Chapter 551). The bacterial enzyme is treated more fully in the following sections.

### Structural Chemistry

The *E. coli* isoaspartyl dipeptidase is encoded by the gene *iadA*, located in the 98 min region of the *E. coli* chromosome (Gary & Clarke, 1995). The *iadA* protein is cytosolic and contains 390 amino acids with a calculated pI of 5.02. The enzyme has a molecular mass of 41 kDa as predicted from its DNA sequence; this size was confirmed by SDS-PAGE analysis (Gary & Clarke, 1995). The isoaspartyl dipeptidase activity elutes as a species of approximately 120 kDa on a Sephadex G200 gel-filtration column (Haley, 1968; cf. Gary & Clarke, 1995), suggesting that the enzyme may exist in a multimeric complex.

### Preparation

Two bacterial isoaspartyl dipeptidase purifications have been reported. Haley (1968) partially purified (110-fold) the dipeptidase from *E. coli* strain B by size-exclusion and anion-exchange chromatography, achieving a specific activity of  $770 \text{ nmol min}^{-1} \text{ mg}^{-1}$  (using L-isoaspartyl-L-leucine as the substrate) with a 34% yield. Gary & Clarke (1995) included an additional phenyl Sepharose chromatography step to achieve a homogeneous preparation (>3000-fold purification) of the enzyme from the wild-type *E. coli* strain MC1000, as well as from strain JDG100, which overexpresses *iadA* at

least 40-fold over MC1000. Both preparations gave a specific activity of  $1900 \text{ nmol min}^{-1} \text{ mg}^{-1}$  (using L-isoaspartyl-L-leucine as the substrate) and a 15% yield.

### Biological Aspects

L-Isoaspartyl residues in proteins arise spontaneously over time from L-aspartyl and L-asparaginyl residues through nonenzymatic, intramolecular isomerization and deamidation reactions (see Figure 502.1; Stephenson & Clarke, 1989; Wright 1991; Clarke *et al.* 1992). Protein function may be impaired due to the resulting kink in the polypeptide backbone. If this damaged protein is not adequately repaired by the L-isoaspartyl/D-aspartyl methyltransferase, it may be degraded (for a review see Visick & Clarke, 1995). Isoaspartyl-containing peptides can arise from the incomplete degradation of these damaged proteins, because most cellular proteases do not recognize the isoaspartyl linkage (Haley *et al.*, 1966; Murray & Clarke, 1984; Johnson & Aswad, 1990).

In *E. coli*, which has a minimal doubling time of 20 min, the accumulation of isoaspartyl residues may be insignificant during exponential growth. However, after a prolonged period in stationary phase the level of these damaged residues may increase along with their isoaspartyl dipeptide degradation products. The isoaspartyl dipeptidase may prevent the accumulation of these dipeptides, which may be toxic to the bacteria or may result in the depletion of the pool of amino acids necessary for survival in stationary phase (Mandelstam, 1958, 1960; Reeve *et al.*, 1984a,b). These hypotheses were tested in a strain of *E. coli* lacking the *iadA* gene, JDG11000 (Gary & Clarke, 1995). The null mutant strain did not exhibit any obvious phenotypes; exponential growth and stationary phase survival were similar to the parent strain (Gary & Clarke, 1995). In addition, radiolabeling studies did not reveal any accumulation of isoaspartyl-leucine dipeptides (Gary & Clarke, 1995). However, an assay of the null mutant cytosol did show the presence of a secondary isoaspartyl dipeptidase activity that accounts for approximately 31% of the total isoaspartyl dipeptidase activity in crude extracts of the parent strain (Gary & Clarke, 1995). It is possible that the remaining activity is sufficient to compensate for the loss of *iadA* activity; only a strain with both enzymes deleted would then show a phenotype due to isoaspartyl dipeptide accumulation. Preliminary results from a partial purification of this second isoaspartyl dipeptidase activity indicate that its specificity is very similar to that of *iadA*: it hydrolyzes isoaspartyl-leucine but uses neither aspartyl-leucine nor isoaspartyl-glycine as substrates (J.D. Gary, unpublished results).

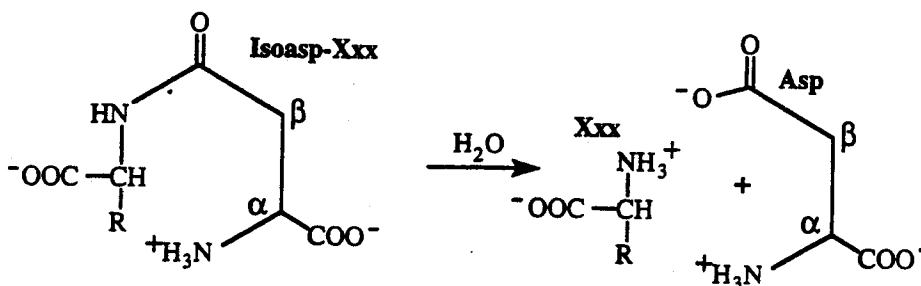
### Distinguishing Features

A database search for protein sequences similar to the *E. coli* isoaspartyl dipeptidase amino acid sequence (performed in July 1996) using the NCBI BLAST program (Altschul *et al.*, 1990) did not identify any related proteases or peptidases. However, significant amino acid sequence similarity to the family of bacterial dihydroorotases was found. The region of highest similarity is within the first 100 amino acids (Figure 502.2) and centers around a ten amino acid region of

1	M	I	D	Y	T	A	A	G	F	T	L	L	Q	G	A	H	L	Y	A	P	E	D	R	G	I	C	D	V	L	V	<i>E. coli</i> isoaspartyl dipeptidase	
1	M	G	V	W	L	K	N	G	M	S	F	N	K	D	G	E	L	M	R	T	H	I	K	-	-	-	-	-	-	-	-	<i>B. caldolyticus</i> dihydroorotase
1	M	S	Y	L	I	K	N	G	W	I	L	N	E	N	G	E	K	T	Q	A	D	I	R	-	-	-	-	-	-	-	<i>B. subtilis</i> dihydroorotase	
31	A	N	G	K	I	I	A	V	A	S	N	I	P	S	D	I	V	P	N	C	T	V	V	D	L	S	G	Q	I	L	<i>E. coli</i> isoaspartyl dipeptidase	
25	E	H	G	T	I	A	A	I	L	Y	E	Q	P	L	E	A	-	-	N	E	D	V	I	D	V	G	G	R	L	I	<i>B. caldolyticus</i> dihydroorotase	
25	T	G	E	T	I	T	A	I	-	-	-	G	K	L	D	A	T	D	N	E	T	V	I	D	A	K	G	L	L	V	<i>B. subtilis</i> dihydroorotase	
61	C	P	G	F	I	D	Q	H	V	H	L	I	G	G	G	E	A	G	P	T	T	R	T	P	E	V	A	L	S	<i>E. coli</i> isoaspartyl dipeptidase		
53	V	P	G	L	I	D	L	H	V	H	L	R	E	P	G	G	E	A	K	E	T	I	E	T	G	T	L	A	A	A	<i>B. caldolyticus</i> dihydroorotase	
52	S	P	G	F	V	D	L	H	V	H	F	R	E	P	G	G	E	K	K	E	T	I	E	T	G	A	K	A	A	G	<i>B. subtilis</i> dihydroorotase	
91	R	L	T	E	A	G	V	T																			<i>E. coli</i> isoaspartyl dipeptidase					
83	K	G	G	F	T	T	V	A																			<i>B. caldolyticus</i> dihydroorotase					
82	R	G	G	Y	T	T	V	A																			<i>B. subtilis</i> dihydroorotase					

Figure 502.2 The N-terminal region of the *E. coli* isoaspartyl dipeptidase sequence aligned with dihydroorotases from *Bacillus caldolyticus* and *Bacillus subtilis*. Residues identical to the dipeptidase are boxed, and the region of highest similarity is shaded. Over the entire protein sequence the dipeptidase is 13% identical in sequence to the dihydroorotases, but this N-terminal region demonstrates 25% identity.

### Isoaspartyl Dipeptidase



### Dihydroorotase

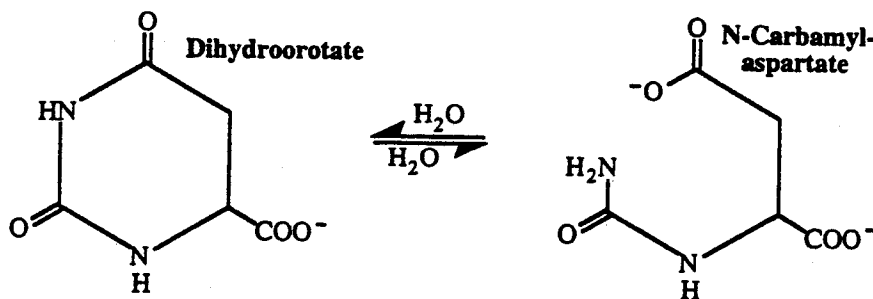


Figure 502.3 Comparison of the reactions catalyzed by the isoaspartyl dipeptidase (top) and by dihydroorotase (bottom), showing the similarity of the reactions and the structural similarity of the substrates.

the dipeptidase with the sequence PGFIDQHVL (see shaded region in Figure 502.2). Studies on the *E. coli* dihydroorotase suggest that this region is a zinc-binding motif, with the two conserved histidines serving as ligands for a catalytic zinc ion (Washabaugh & Collins, 1984, 1986; Brown & Collins, 1991). The observed sequence similarity between the isoaspartyl dipeptidase and dihydroorotases is consistent with a similarity in their substrates and in the chemistry of the reaction catalyzed (Figure 502.3). Isoaspartyl dipeptides and

dihydroorotate have very similar molecular geometries and functional groups, and hydrolysis occurs at a similar position on each molecule. The *E. coli* isoaspartyl dipeptidase, however, has no dihydroorotase activity (Gary & Clarke, 1995). It appears that in order to cleave an abnormal type of peptide bond, *E. coli* cells have evolutionarily derived a unique dipeptidase from an unrelated metabolic enzyme rather than modifying an existing peptidase from an  $\alpha$ - to a  $\beta$ -carboxyl specificity.

## References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **21**, 403–410.
- Brown, D.C. & Collins, K.D. (1991) Dihydroorotase from *Escherichia coli*. Substitution of Co(II) for the active site Zn(II). *J. Biol. Chem.* **266**, 1597–1604.
- Buchanan, D.L., Haley, E.E., Markiw, R.T. & Peterson, A.A. (1962) Occurrence of  $\beta$ -aspartyl and  $\gamma$ -glutamyl oligopeptides in human urine. *Biochemistry* **1**, 612–620.
- Clarke, S., Stephenson, R.C. & Lowenson, J.L. (1992) Lability of asparagine and aspartic-acid residues in proteins and peptides: spontaneous deamidation and isomerization. In: *Stability of Protein Pharmaceuticals, Part A* (Ahern, T.J. & Manning, M.C., eds). New York: Plenum, pp. 1–29.
- Dorer, F.E., Haley, E.E. & Buchanan, D.L. (1966) Quantitative studies of urinary beta-aspartyl oligopeptides. *Biochemistry* **5**, 3236–3240.
- Dorer, F.E., Haley, E.E. & Buchanan, D.L. (1968) The hydrolysis of  $\beta$ -aspartyl peptides by rat tissue. *Arch. Biochem. Biophys.* **127**, 490–495.
- Gary, J.D. & Clarke, S. (1995) Purification and characterization of an isoaspartyl dipeptidase from *Escherichia coli*. *J. Biol. Chem.* **270**, 4076–4087.
- Grassmann, W. & Schneider, F. (1934) Zur Spezifität der Dipeptidase. Enzymatisches Verhalten von Asparaginsäure- und Glutaminsäurepeptiden [On the specificity of dipeptidases. Enzymatic action on asparagine and glutamine peptides]. *Biochem. Z.* **273**, 452–465.
- Greenstein, J.P. & Price, V.E. (1949)  $\alpha$ -Keto acid-activated glutaminase and asparaginase. *J. Biol. Chem.* **178**, 695–705.
- Haley, E.E. (1968) Purification and properties of a  $\beta$ -aspartyl peptidase from *Escherichia coli*. *J. Biol. Chem.* **243**, 5748–5752.
- Haley, E.E., Corcoran, B.J., Dorer, F.E. & Buchanan, D.L. (1966)  $\beta$ -aspartyl peptides in enzymatic hydrolyzates of protein. *Biochemistry* **5**, 3229–3235.
- Hanson, H.T. & Smith, E.L. (1949) Carnosinase: an enzyme of swine kidney. *J. Biol. Chem.* **179**, 789–801.
- Johnson, B.A. & Aswad, D.W. (1990) Fragmentation of isoaspartyl peptides and proteins by carboxypeptidase Y: release of isoaspartyl dipeptides as a result of internal and external cleavage. *Biochemistry* **29**, 4373–4380.
- Mandelstam, J. (1958) Turnover of protein in growing and non-growing populations of *Escherichia coli*. *Biochem. J.* **69**, 110–119.
- Mandelstam, J. (1960) The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. *Bacteriol. Rev.* **24**, 289–308.
- Murray, E.D., Jr. & Clarke, S. (1984) Metabolism of a synthetic L-isoaspartyl-containing hexapeptide in erythrocyte extracts: enzymatic methyl esterification is followed by nonenzymatic succinimide formation. *J. Biol. Chem.* **259**, 10722–10732.
- Reeve, C.A., Bockman, A.T. & Matin, A. (1984a) Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **157**, 758–763.
- Reeve, C.A., Amy, P.S. & Matin, A. (1984b) Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *J. Bacteriol.* **160**, 1041–1046.
- Riniker, B. & Schwyzer, R. (1964) Synthetische Analoge des Hypertensins.  $\alpha$ -L-,  $\beta$ -L-,  $\alpha$ -D- und  $\beta$ -D-Asp-Val-Hypertensin II; desamino-Val-Hypertensin II [Synthetic analogs of the hypertensins.  $\alpha$ -L-,  $\beta$ -L-,  $\alpha$ -D- and  $\beta$ -D-Asp-Val-Hypertensin II; desamino-Val-Hypertensin II]. *Helv. Chim. Acta* **47**, 2357–2374.
- Stephenson, R.C. & Clarke, S. (1989) Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. *J. Biol. Chem.* **264**, 6164–6170.
- Visick, J.E. & Clarke, S. (1995) Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. *Mol. Microbiol.* **16**, 835–845.
- Washabaugh, M.W. & Collins, K.D. (1984) Dihydroorotase from *Escherichia coli*: purification and characterization. *J. Biol. Chem.* **259**, 3293–3298.
- Washabaugh, M.W. & Collins, K.D. (1986) Dihydroorotase from *Escherichia coli*: sulfhydryl group-metal ion interactions. *J. Biol. Chem.* **261**, 5920–5929.
- Wright, H.T. (1991) Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit. Rev. Biochem. Mol. Biol.* **26**, 1–52.

Jonathan D. Gary

Molecular Biology Institute and  
Department of Chemistry and Biochemistry,  
University of California, Los Angeles,  
Los Angeles, CA 90095-1569, USA

Steven Clarke

Molecular Biology Institute and  
Department of Chemistry and Biochemistry,  
University of California, Los Angeles,  
Los Angeles, CA 90095-1569, USA  
Email: [clarke@ewald.mbi.ucla.edu](mailto:clarke@ewald.mbi.ucla.edu)