Effect of Salt on the Activity and Stability of Aspartate Aminotransferase from the Halophilic Archaebacterium *Haloferax mediterranei*

Francisco J. G. Muriana[a], María C. Alvarez-Ossorio[a], María M. Sánchez-Garcés[a], Francisco F. de la Rosa[b], and Angel M. Relimpio[a]

[a] Departamento de Bioquímica, Facultad de Farmacia, Universidad de Sevilla
[b] Instituto de Bioquímica Vegetal y Fotosíntesis, Facultad de Biología, Universidad de Sevilla y C.S.I.C., Sevilla, Spain

Z. Naturforsch. **47c**, 375–381 (1992); received December 17, 1991/February 21, 1992

Halophilic Bacteria, Aspartate Aminotransferase, Salt Effects, Activity and Stability, *Haloferax mediterranei*

The aspartate aminotransferase from *Haloferax mediterranei*, which is in the cell mainly as apoenzyme, requires high concentrations of salt for both activity and stability. The maximum activity is reached with 3.5 M KCl in the assay. The effect of different cations and anions has been studied using several types of salts. Monovalent cations show a significant difference in effectiveness of promoting the activity with the following order: $K^+ > Rb^+ > Na^+ > NH_4^+$. Mg^{++} and polyvalent cations with organic character cause partial activity with maximum effectiveness at 0.1 M, an inhibition at higher concentration is observed. Anions, added as potassium salts, promote enzyme activity with the following order: $Cl^- > NO_3^- > I^- > SCN^-$. Like activity, the enzyme stability depends on salt concentrations. Incubation of the enzyme with a low salt concentration leads to inactivation following pseudofirst order kinetics. The inactivated enzyme is partially reactivated by high concentrations of KCl following second order kinetics. Taking into account the dimeric structure of this enzyme, high concentrations of salt could stabilize the dimer, which is the active form. The salt effects on halophile aspartate aminotransferase are discussed considering hydrophobic and electrostatic interactions.

Introduction

Enzymes from halophilic bacteria are characterized by the requirement of a high salt concentration to maintain their physic-chemical properties, thus, the stability and maximum activity of these enzymes are usually reached only with a high salt concentration, about 3 M of KCl or NaCl.

In general, the halophilic enzymes, with the exception of dehydrogenases, have been studied very little. The purification of halophilic dehydrogenases carried out by Mevarech *et al.* [1] and Leicht *et al.* [2] have allowed the posterior analysis of their molecular structures [1, 3] and the conformational changes produced by the interaction with different salts [3–6].

The halophilic aminotransferases are also almost unknown. No attention has been paid to these enzymes until we characterized the aspartate aminotransferase from *Haloferax mediterranei* [7]. The AspAt from this halophilic bacteria, in contrast to the enzyme from animals, which is obtained mainly as holoenzyme, is present in the cell in both apo (60%) and holo (40%) forms [7].

AspAT from animals and *Escherichia coli* is a dimer with two identical subunits [8]. The enzyme needs PLP for catalysis and its dimeric structure is protected by the coenzyme against thermal denaturation [9, 10] or against dissociation by urea [11] or guanidine [12].

In preparations of aminotransferases from animals, it has been observed that the coenzyme is separated from the apoenzyme as pyridoxamine easier than as pyridoxal by a relatively high concentration of ammonium sulphate [13] or phosphate [14, 15]. This is due to the fact that PMP, but not PLP, is bound to the protein mainly by electrostatic interactions [16]. Glutamate decarboxylase shows a similar process where the activity is regulated, through the equilibrium between holoenzyme and apoenzyme, by phosphate concentration in the cell [17].

Abbreviations: hAspAT, halophilic aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate

Reprint requests to Francisco F. de la Rosa. Instituto de Bioquímica Vegetal. Facultad de Biología. Universidad de Sevilla. Apartado 1095, 41080 Sevilla, Spain.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/92/0500–0375 $ 01.30/0

In this paper, the effect of salts on apo and holo forms of AspAT from *Haloferax mediterranei* will be examined.

Materials and Methods

Chemicals

NADH, pyridoxal 5′-phosphate (PLP), L-aspartate, α-ketoglutarate, malate dehydrogenase (pig heart), spermine, lysine and cysteine were from Sigma Chemical Co. 1,6-diaminohexane and 1,3-diaminepropane were from Merck. All other chemicals used were of analytical grade or the finest grade available.

Bacterial strains and growth

The strain ATCC 33500 of *Haloferax mediterranei* used in this work was kindly provided by Dr. Ruiz-Berraquero from the University of Sevilla. The halophilic bacterium was grown aerobically in the medium described by Rodriguez-Valera *et al.* [18]. After three days of growth, the cells were collected by centrifugation and resuspended in a medium containing 0.01 sodium phosphate (pH 7.2) and 3 M NaCl.

Enzyme activity

hAspAT was obtained following the method described by Muriana *et al.* [7]. Different enzyme concentrations were obtained by dilution from a preparation maintained in 1 M ammonium sulfate. The concentration of ammonium sulfate after dilution was in any case lower than 20 mM. The activity was measured by coupling the oxalate production with malate dehydrogenase and following the NADH oxidation at 340 nm. Enzymatic assays were performed in 1 ml of the standard buffer solution (3.1 M KCl and 50 mM Tris/HCL) containing, additionally, 10 mM α-ketoglutarate, 0.3 mM NADH, 60 IU malate dehydrogenase, 0.05 mM PLP and the AspAT. The reaction was started by the addition of 100 μM L-aspartate. The oxidation of NADH was measured at 340 nm in a Philips spectrophotometer PU 8620. The enzymatic activity is expressed in international units (IU).

Results

Effects of different salts on the activity of hAspAT

The hAspAT shows maximum activity when a high concentration of salt is present in the assay. In Fig. 1, the effect of monovalent cations, added as chloride salts, is shown. K^+ appears as the most effectively promoting cation, reaching maximum activity at 3.5 M. The effectiveness of cations shows the following order: $K^+ > Rb^+ > Na^+ > NH_4^+$.

The action of Mg^{2+} and polyvalent cations with organic character is shown in Fig. 2. The low concentrations used in this case were due to the low solubility of these ions. Maximum activity is reached with 0.07 M Mg^{2+}, which corresponds to 50% of the activity in the presence of 3 M K^+. When Mg^{2+} is added in the presence of spermine the activity promoted is lower than with the polyvalent cation alone. However, Mg^{2+} increases the activity observed with K^+ (data not shown).

In Fig. 3, the effect of several anions added as potassium salts is shown. In this case, the effectiveness shows the following order: $Cl^- > NO_3^- > I^- > SCN^-$. That coincides with the order of Hofmeister series for the hydrophobic interactions.

The affinity of the hAspAT for the substrates is also affected by the concentration and nature of salts. Thus, the K_m for both aspartate and α-ketoglutarate is lower in the presence of KCl than with NaCl and increases proportionally to the KCl concentration (Table I).

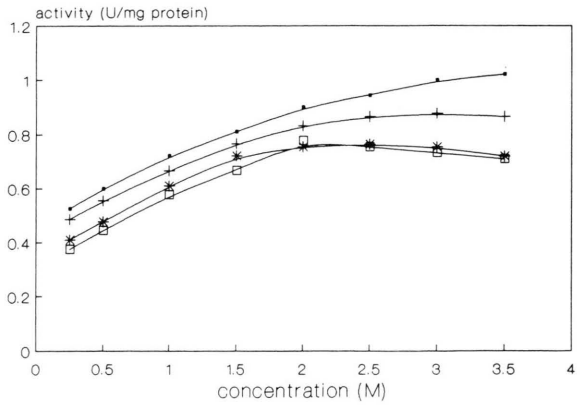

Fig. 1. Effect of different monovalent cations on the activity of hAspAT. The activity was assayed as indicated in Materials and Methods except that the concentrations of the following salts indicated in the figure were used: KCl (■), NaCl (★), NH_4Cl (□) and RbCl (+). The data correspond to the average of three experiments.

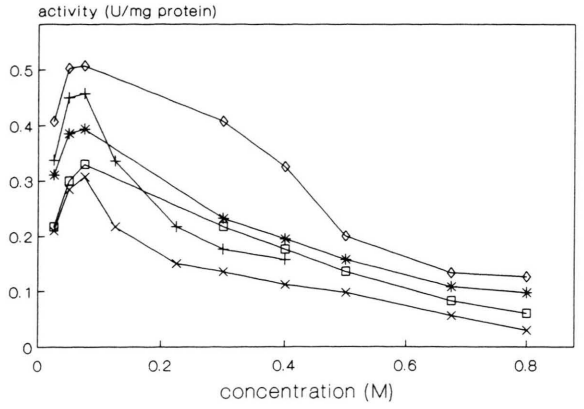

Fig. 2. Effect of polivalent cations on the activity of hAspAT. The activity was determined as indicated in Materials and Methods. The following salts at the indicated concentrations were added to the assay cuvette: $MgCl_2$ (◇), 1,6-diaminehexane (★), 1,3-diaminepropane (□), spermine (+), $MgCl_2$ plus spermine (×). The data correspond to the average of three experiments.

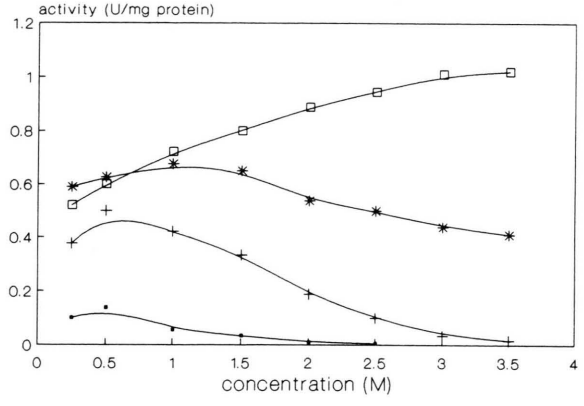

Fig. 3. Effect of several anions on the activity of hAspAT. The activity was assayed as indicated in Materials and Methods in the presence of the following salts at the indicated concentrations: KSCN (■), KI (+), KNO_3 (★) and KCl (□). The data correspond to the average of three experiments.

Effect of salt concentration on the stability of hAspAT

The hAspAT is rapidly inactivated when incubated with a low concentration of salt. In Fig. 4, the inactivation in the presence of different concentrations of KCl is shown. The inactivation follows a pseudo-first order kinetic, with a halflife of 8.6 h (k_i = 0.08 h^{-1}) for a concentration of 1.5 M and 1.8 h (k_i = 0.375 h^{-1}) for 0.5 M.

Table I. Dependence of Michaelis constant of halophilic aspartate aminotransferase on the concentration of KCl and NaCl.

	K_m [mM]	
	Asp	K−Glu
KCl [M]		
0.7	2 ± 0.2	0.9 ± 0.1
1.5	3.2 ± 0.2	1.7 ± 0.1
3.5	5.5 ± 0.1	2.5 ± 0.1
NaCl [M]		
3.5	10.0 ± 0.2	2.7 ± 0.2

The fixed concentration, when the other substrate was variable, for aspartate was 100 mM and for α-ketoglutarate 10 mM. Other experimental conditions as indicated in Materials and Methods. The data are the average from at least three experiments.

In Fig. 5, it is shown the logarithmic relation between inactivation rate constant (k_i) and concentration of KCl and NaCl, which indicates a sigmoidal dependence of the activity on salt concentration. The values for Hill's coefficient (n) obtained from the slopes were 1.33 for KCl and 0.24 for NaCl.

Like KCl and NaCl, ammonium sulphate proved to be a good stabilizer of hAspAT maintaining the activity during several months [19].

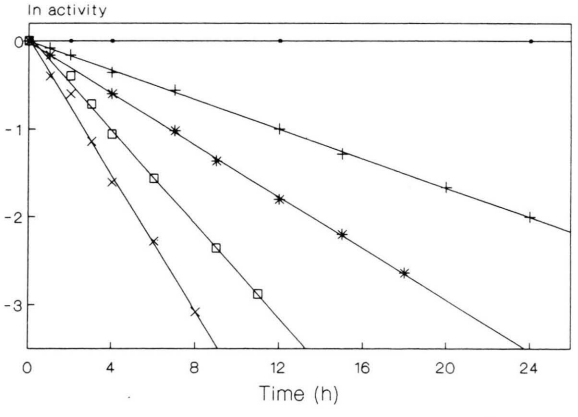

Fig. 4. Inactivation of the hAspAT by several concentrations of KCl. Several preparations of the hAspAT were incubated at 4 °C in the presence of KCl at the following concentrations: 3 (■), 1.5 (+), 1.25 (★), 1 (□) and 0.5 M (×). The activity was measured on aliquots obtained at different times as indicated in Materials and Methods. One hundred per cent corresponds to a preparation incubated at the same temperature with 3.5 M KCl.

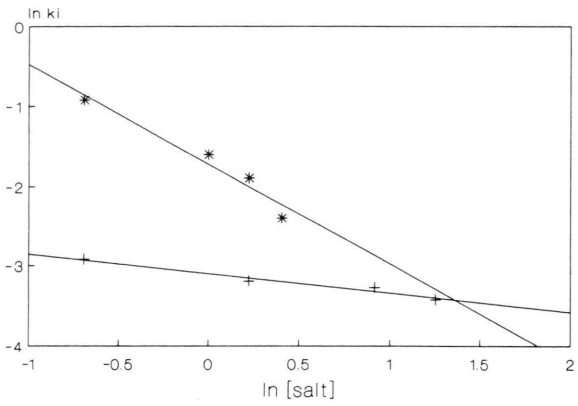

Fig. 5. Logarithmic plot of k_i from the inactivation process of the hAspAT and the concentrations of the salts KCl and NaCl. Several preparations of the hAspAT were incubated in the presence of 50 mM Tris/HCl (ph 7.8) with KCl (★) or NaCl (+) at the concentrations indicated in the figure. The values of the pseudofirst order apparent constant were determined from the semilogarithmic plot and expressed in hours.

Table II. Dependence of inactivation and reactivation of the halophilic aspartate aminotransferase on the KCl concentration.

KCl [M]	E_i	E_a	Non-reactivated enzyme
3.5	100	100	0
2.5	68.5	96.8	3.2
2.0	61.4	95.3	4.7
1.0	22.0	80.1	19.9
0.5	14.3	75.0	25.0
0.0	11.4	51.3	48.7

Preparations of hAspAT were dialyzed for 12 h against the concentrations of KCl indicated. The activity was then measured and indicated as E_i. Finally, the preparations were dialyzed over-night against 3.7 M KCl and the activity measured and expressed as E_a.

The reversibility of the inactivation has been studied in preparations of the active enzyme inactivated by dialysis for 12 h against a low concentration of salts and then incubated with 3.5 M KCl (Table II). A partial reactivation is observed which depends in each case of the amount of inactivation reached. The irreversible inactivation is observed by precipitation of the protein which is general in the absence of any type of salt.

The reactivation process follows a second order kinetic as observed in Fig. 6. This suggests, taking into account the dimeric structure of the AspAT, a reassociation of monomers during the enzyme reactivation. Thus, the reactivation constant (k_r) can be determined, as indicated by Mevarech and Neumann [3], from the equation:

$$\frac{[E_{a,t}]}{4\,[E_{a,\infty}]\cdot([E_{a,\infty}] - [E_{a,t}])} = k_r \cdot t \qquad (1)$$

where $[E_{a,t}]$ and $[E_{a,\infty}]$ are, respectively, the concentration of the active enzyme at time t and the concentration when no further reactivation occurs. This analysis has the advantage that it is not necessary to know the initial enzymatic activity.

Fig. 6 inset shows the values of the above equation *versus* time for a preparation inactivated at 1 M KCl and reactivated by incubation with 3.5 M KCl. A reactivation rate constant, k_r, equal to $4.7 \times 10^{-5}\ M^{-1}\ s^{-1}$ is obtained from the slope.

The results described above have been carried out with the apoenzyme because of the low concentration of PLP present in the preparations. With the idea of checking the behavior of the holoenzyme, the same studies were made in the presence of 0.05 mM PLP. Under these conditions, since the dissociation constant for PLP is about 7 μM [7], the enzyme remains mainly as holoenzyme. The results obtained with holoenzyme were almost similar (Fig. 7), although, the activity that

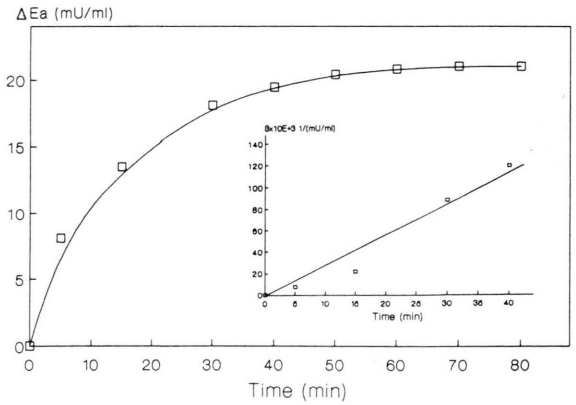

Fig. 6. Reactivation kinetics of the hAspAT. A preparation of the enzyme was inactivated as indicated in Materials and Methods by dialysis against 2.5 M KCl. The reactivation was carried out by incubation with 3.7 M KCl at 25 °C. The increase of activity (△ Ea) at different times of reactivation is expressed in the figure. The inset shows the analysis of the reactivation of the enzyme as indicated by Mevarech and Neumann [3] (see text); β corresponds to the values obtained from Eq. (1).

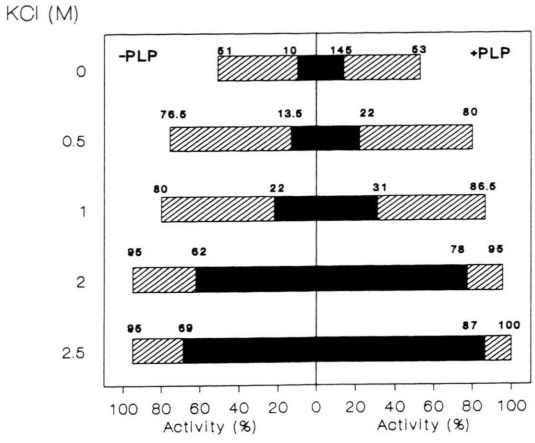

Fig. 7. Reactivation by different concentrations of KCl of the inactivated hAspAT in both apo and holo enzyme forms. Preparations of hAspAT were inactivated by incubation at low concentrations of KCl in the presence and in the absence of 0.05 mM PLP. The reactivation was carried out by incubation with 3.7 M KCl for 8 h at 4 °C both with and without PLP. The activity before and after reactivation is shown on the left without PLP and on the right with PLP.

remains after the inactivation in the holoenzyme is a little higher.

Discussion

In the studies on the effect of different salts on the hAspAT, a promotion of the enzyme activity by monovalent cations has been observed, among them K^+ shows the highest efficiency. The enzyme activity presents a sigmoidal dependence on salt concentration with a Hill's coefficient of 1.33 and 0.24 for KCl and NaCl respectively. This difference indicates a higher sensitivity of hAspAT to changes in the concentration of K^+ than to Na^+. A similar behavior, although with higher Hill's coefficients, was found with other halophilic enzymes, such as pyruvate kinase [20], isocitrate dehydrogenase [21] and citrate synthase [22]. This different effect for K^+ and Na^+ agrees with the different concentrations observed in the cell for these ions [23, 24], which suggests a regulation of the activity by the ratio of concentrations of these ions in the cell.

Mg^{2+} and polyvalent cations have shown a lower effectiveness in the promotion of the activity, showing an inactivation effect for concentrations higher than 0.05 M (Fig. 2).

Like activity, stability of hAspAT needs a high concentration of salt (Fig. 4). It is interesting to note that with hAspAT the activity and the stability showed a parallel dependence on the salt concentration, the maxima activity and stability found at 3 M KCl. This represents a difference in comparison with other halophilic enzymes such as malate dehydrogenase which presents a maximum activity at 1.25 M NaCl whereas the stability is maximum at a concentration of 3 M [2].

The effects of salt on the activity and structure of halophilic enzymes has been discussed taking into account the electrostatic and hydrophobic interactions, although the competition in the formation of hydrate bonds between protein-solvent and protein-protein as the most important contributing factor to polypeptide folding of these proteins has been proposed recently [25, 26].

In principle, the effect of cations on hAspAT could be explained by electrostatic interactions. Thus, the action of monovalent cations is explained by the interactions with the charged amino acids, which in proteins have only one charge and in this enzyme are mainly negative, as deduced from the isoelectric point, 6.1, and amino acid composition [7]. In general, the amino-acid composition of halophilic proteins shows a 10–14 mol% excess of negative charges when compared with non-halophilic proteins [27]. The difference among monovalent cations observed by us must be due to the different size of ions that produce steric impediment in the accessibility to neutralize groups into the protein. In this way, the order of effectiveness observed ($K^+ > Rb^+ > Na^+ > NH_4^+$) coincides with the hydrated radius of cations [28].

In contrast to electrostatic interactions as a main factor of stabilization is the high concentration that is necessary for the maximum activity (3 M). Usually, the action by the ionic charge is saturated with a concentration of 0.2 M [29–31].

The low effect observed by Mg^{2+} and polyvalent cations could be explained, taking into account electrostatic interactions, by the necessity of coincidence of two or more charge groups in the area of influence of the cation.

The hydrophobic interactions must play a main role in the stability of hAspAT as seems to occur in other halophilic proteins [26]. The effect of cations observed in our case is in opposition with the Hofmeister series, which gives of following order for

the hydrophobic interactions ($Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$). Nevertheless, the effect observed by anions coincides with the Hofmeister series ($SO_4^= > Cl^- > NO_3^- > I^- > SCN^-$). This explains the action of ammonium sulphate which is in the first position and maintains the enzymatic activity for several months [19]. This suggests an important hydrophobic effect in the stabilization of the hAspAT.

To sum up, the effect on activity and stability of hAspAT by salts must be due to both electrostatic and hydrophobic interactions and, in consequence, the neutralization of the charges in the interior of molecule (specially considering the character acid of this protein) and the reinforcement of the external hydrophobic bonds (by the increase of entropy in the medium) must play critical roles in these process.

Taking into account the dimeric structure of AspAT and the second order kinetic observed in the reactivation (Fig. 6), the inactivation and reactivation must be coupled to the dissociation and reassociation of monomers. Thus, the free-energy changes associated with the dissociation process may be treated according to the "salting-out" theories [28, 32], where the free-energy change is linearly dependent on the salt concentration [33]. The halophilic malate dehydrogenase shows a similar behavior, its denaturation by low salt concentration involves enzyme-dimer dissociation [26, 34].

The fact that the inactivated hAspAT is only partially reactivated suggests the existence of a second and irreversible conformation of the monomer in agreement with the scheme:

$$E_a \rightleftharpoons 2\,E_i \rightarrow E_i^*$$

which refers to the Lumry and Eyring model [35] and appears frequently in the thermal denaturation of proteins [36, 37].

Finally, the behavior of hAspAT which seems to be similar to other halophilic enzymes suggests the existence of an unique mechanism for the stabilization of the structure of halophilic proteins.

Acknowledgements

This work was sponsored by Grant No. PB86-0167-CO3-02 from Comisión Interministerial de Ciencia y Tecnología, and by a financial help from Plan Andaluz de Investigación.

[1] M. Mevarech, H. Eisenberg, and E. Neumann, Biochemistry **16,** 3781–3785 (1977).
[2] W. Leicht, M. M. Weber, and H. Eisenberg, Biochemistry **17,** 4004–4010 (1978).
[3] M. Mevarech and E. Neumann, Biochemistry **16,** 3786–3792 (1977).
[4] S. Pundak and H. Eisenberg, Eur. J. Biochem. **118,** 463–470 (1981).
[5] K. Hecht, A. Wrba, and R. Jaenicke, Eur. J. Biochem. **183,** 69–74 (1989).
[6] K. Hecht and R. Jaenicke, Biochemistry **28,** 4979–4985 (1989).
[7] F. J. García-Muriana, M. C. Alvarez-Ossorio, and A. M. Relimpio, Biochem. J. **278,** 149–154 (1991).
[8] A. E. Braunstein, in: The Enzymes, 3rd ed. (Boyer, P. D., ed.) **Vol. 9,** pp. 379–481 (1973).
[9] A. M. Relimpio, A. Iriarte, J. F. Chlebowski, and M. Martínez-Carrión, J. Biol. Chem. **256,** 4478–4488 (1981).
[10] A. Iriarte, H. A. Farach Jr, and M. Martínez-Carrión, J. Biol. Chem. **259,** 7003–7010 (1984).
[11] V. I. Ivanov, A. L. Bocharov, M. V. Volkenstein, M. Y. Karpeisky, S. Mora, E. I. Okina, and L. V. Yudina, Eur. J. Biochem. **40,** 519–526 (1973).
[12] M. Herold and K. Kirschner, Biochemistry **29,** 1907–1913 (1990).
[13] H. Wada and E. E. Snell, J. Biol. Chem. **237,** 127–132 (1962).
[14] M. Martínez-Carrión, R. Kuczenski, D. C. Thiemeier, and D. L. Peterson, J. Biol. Chem. **245,** 799–805 (1970).
[15] E. J. Churchich and U. Moses, J. Biol. Chem. **256,** 1101–1104 (1981).
[16] J. N. Jansonius, G. Eichele, G. Ford, D. Picot, C. Thaller, and M. G. Vincent, in: Transaminases (Christen P. and Metzler D. E. eds.), John Wiley, New York 1985.
[17] J. R. Cooper, F. E. Bloom, and R. H. Roth, in: The Biochemical Basis of Neuropharmacology, Chap. 9, Oxford University Press, New York, 1978.
[18] F. Rodriguez-Varela, F. Ruíz-Berraquero, and A. Ramos-Cormenzana, J. Gen. Microbiol. **119,** 535–538 (1980).
[19] F. J. García-Muriana and A. M. Relimpio, J. Gen. Appl. Microbiol. **37,** 315–320 (1991).
[20] E. De Médicis, J. F. Laliberté, and J. Vass-Marengo, Biochim. Biophys. Acta **708,** 57–67 (1982).
[21] D. M. Aitken, A. J. Wicken, and A. D. Brown, Biochem. J. **116,** 125–134 (1970).
[22] A. Higa and J. J. Cazzulo, Biochem. J. **147,** 267–274 (1975).
[23] E. D. Dundas, Adv. Microbiol. Physiol. **15,** 85–120 (1977).
[24] C. R. Woese, Microbiol. Rev. **51,** 221–271 (1987).
[25] G. Zaccai and H. Eisenberg, Trends Biochem. Sci. **15,** 333–337 (1990).

[26] H. Eisenberg, Eur. J. Biochem. **187,** 7–22 (1990).
[27] H. Eisenberg and E. J. Wachtel, Ann. Rev. Biophys. Chem. **16,** 69–92 (1987).
[28] F. A. Cotton and G. Wilkinson, in: Advanced Inorganic Chemistry, 4 Edition, Table 7-2, John Wiley & Sons Inc. New York 1981.
[29] J. K. Lanyi, J. Biol. Chem. **244,** 2864–2869 (1969).
[30] J. K. Lanyi, J. Biol. Chem. **244,** 4168–4173 (1969).
[31] J. K. Lanyi and J. Stevenson, J. Biol. Chem. **245,** 4074–4080 (1970).
[32] E. E. Schrier and E. B. Schrier, J. Phys. Chem. **71,** 1851–1856 (1967).
[33] Y. Hatefi and W. G. Hanstein, Proc. Nat. Acad. Sci. U.S.A. **62,** 1129–1136 (1969).
[34] S. Pundak, H. Aloni, and H. Eisenberg, Eur. J. Biochem. **18,** 471–477 (1981).
[35] R. Lumry and H. Eyring, J. Phys. Chem. **58,** 110–115 (1954).
[36] A. M. Klibanov and T. J. Ahern, in: Protein Engineering (D. L. Oxender and C. F. Fox eds.) Liss, New York 1987.
[37] M. L. Galisteo, P. L. Mateo, and J. M. Sánchez-Ruiz, Biochemistry **30,** 2061–2066 (1991).