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Thermodynamic parameters for the glutamate dehydrogenase catalyzed α-imino acid–α-amino acid interconversion

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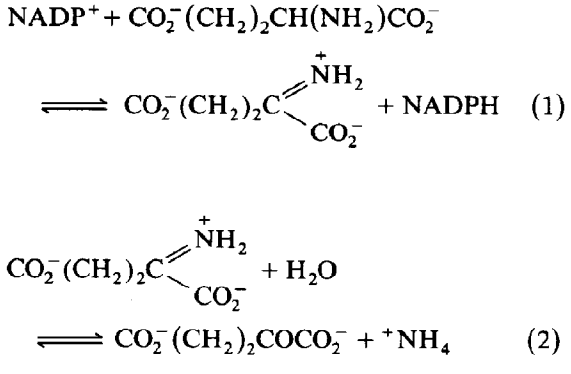
Δ^1-Piperidine 2-carboxylic acid, an α-imino acid, is reduced by 1,4-dihydropyridines to pipecolic acid, an α-amino acid, and the corresponding pyridinium ions. This nonenzymatic reaction occurs only in the direction of pipecolic acid production. Glutamate dehydrogenase catalyzes this reaction when the reductant is NADPH and gives as products L-pipecolic acid and $NADP^+$. The reaction velocity for the enzyme-catalyzed reaction is measurable in either direction. The pH-independent equilibrium constant, K_{eq}, for the reduction of the imino acid by NADPH to give pipecolic acid anion and $NADP^+$ was determined from the equilibrium conditions and the pK_a values of pipecolic acid (10.72) and of the cyclic imino acid (8.10). The value of K_{eq} was found to be 175 ± 30; the values of ΔG°, ΔH° and ΔS° are -3.1 ± 0.1 kcal/mol, 5 ± 1 kcal/mol and 27 ± 4 e.u., respectively. The data indicate that the reactants are far more solvated than the products and that there must be a large degree of solvent reorganization during the course of the reaction. If these thermodynamic parameters apply to the redox step of the enzyme-catalyzed glutamate reaction, then the burst phase which results upon mixing the enzyme, L-glutamate and $NADP^+$ in stoichiometric amounts must contain a hidden nonredox step of large ΔH° value to account for the curved Arrhenius plot observed for this phase (A.H. Colen, R.T. Medary and H.F. Fisher, Biopolymers 20 (1981) 879).

1. Introduction

Although dehydrogenases catalyze the net transfer of hydride ion from their substrates to oxidized coenzymes, this step is never solely rate-limiting except for the formate dehydrogenase catalyzed oxidation of formate [1]. In the case of glutamate dehydrogenase, which catalyzes the reversible oxidative deamination of L-glutamate by $NADP^+$ (eqs 1 and 2), the redox step is at best partially rate limiting under any experimental conditions that have thus far been studied.



$$NADP^+ + CO_2^-(CH_2)_2CH(NH_2)CO_2^- \rightleftharpoons CO_2^-(CH_2)_2C(=NH_2^+)CO_2^- + NADPH \quad (1)$$

$$CO_2^-(CH_2)_2C(=NH_2^+)CO_2^- + H_2O \rightleftharpoons CO_2^-(CH_2)_2COCO_2^- + {}^+NH_4 \quad (2)$$

Thus, the slow step under steady-state conditions is the release of products from enzyme ternary complexes in the forward direction [2], while that in the reverse direction is not well characterized.

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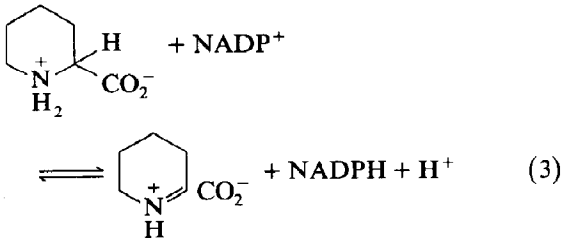
Although Iwatsubo and Pantaloni [3] had observed a burst of NADPH production when stoichiometric amounts of glutamate dehydrogenase were mixed with L-glutamate and $NADP^+$, this burst phase appears to include the hydride transfer event and the subsequent imine hydrolysis step. Jallon et al. [4] observed a burst of NADPH production in the reverse direction — the reductive amination of α-ketoglutarate, but this burst phase includes both the imine formation and NADPH oxidation steps.

The redox step with model substrates is more rate-limiting than that with L-glutamate. This is the case for the oxidation of the acyclic substrate, norvaline, by enzyme-$NADP^+$ [5]. However, the resulting imino acid is hydrolyzed to the corresponding α-keto acid and ammonia under the experimental conditions, and the hydrolysis step makes an unknown extent of contribution to the observed rate. Srinivasan and Fisher [6] found that the oxidation of L-proline by $NADP^+$ to give the cyclic imino acid, Δ^1-pyrroline 2-carboxylic acid (PCA) and NADPH is catalyzed by glutamate dehydrogenase. The hydride transfer step is at least significantly rate-limiting with proline. Since the product imino acid does not undergo hydrolysis under the experimental conditions, the rate data are free from contributions from that step. However, only a portion of the normal catalytic machinery of the enzyme was expressed in this redox process.

As part of our effort to understand the redox step of the enzyme-catalyzed oxidative deamination of L-glutamate, we have investigated the glutamate dehydrogenase catalyzed interconversion of L-pipecolic acid — Δ^1-piperidine 2-carboxylic acid (PPA) (eq. 3)



(3)

Although the structures of PCA and PPA are quite similar, their properties differ in some important respects. For example, they have vastly different pK_a values, 6.05 for PCA [7] and 8.10 for PPA, and the kinetic deuterium isotope effects for the reduction of the cyclic imino acids by enzyme-NADH and enzyme-NADH-4,4d_2 are greater than 1 for PCA [7] and inverse for PPA (unpublished results from our laboratory). Consequently, an investigation of the reaction shown in eq. 3 became necessary.

In this report, we present the thermodynamic constants for the model reaction shown in eq. 3 and examine the implications of the data to the glutamate dehydrogenase catalyzed oxidative deamination of L-glutamate.

2. Experimental

β-NADPH (Boehringer-Mannheim), α-ketoglutaric acid (Sigma), potassium phosphate (Mallinkrodt) and β-$NADP^+$ (Sigma) were used as supplied. L-Pipecolic acid and its DL isomer were purchased from Aldrich. Prior to use, the amino acids were purified by repeated crystallization. L-Amino acid oxidase and catalase were supplied by Sigma. 1-Benzyl-3-carbamoylpyridinium chloride and 1-benzyl-3-carbamoyl-1,4-dihydropyridine were synthesized according to published procedures [8].

2.1. Δ^1-Piperidine-2-carboxylic acid (PPA)

This compound was synthesized by a modification [9] of the procedure of Meister [10]. The synthesis involves the preparation of *N*-ε-aminocarbobenzoxylysine which is subsequently oxidized to α-ketocarbobenzoxylysine by L-amino acid oxidase. The carbobenzoxy group is later removed to produce ε-amino-α-ketocaproic acid. This compound spontaneously cyclizes to the hydrobromide salt of PPA, which upon recrystallization from ethanol-ether produced colorless crystals, m.p. 190–191° C (ref. 9: m.p. 190° C).

Anal. Calculated for the anhydrous compound $C_6H_{10}NO_2Br$: C, 34.62; H, 4.81; N, 6.73; Br, 38.46. Found: C, 34.56; H, 5.01; N, 6.74; Br, 38.47.

The absorption spectrum of the compound in 0.1 M NaOH was identical to that obtained by Roseman [9].

2.2. L-Glutamate dehydrogenase

Bovine liver L-glutamate dehydrogenase was purchased as an ammonium sulfate suspension from Boehringer-Mannheim. Before use, the enzyme was exhaustively dialyzed against 0.1 M potassium phosphate buffer, pH 7.6, and treated with charcoal to remove tightly bound organic impurities [11]. The concentration of the enzyme was determined by measuring its absorbance at 280 nm using an absorptivity of 0.97 cm^{-1} mg^{-1} ml and $M_w = 56100$ [12].

The absorbance of NADPH at 340 nm ($\epsilon_{340} = 6300$ M^{-1} cm^{-1} [13]) was used to calculate its concentration. $NADP^+$ concentrations were calculated using $\epsilon_{259} = 17800$ [14].

2.3. Reaction products

The hydrobromide salt of PPA (0.37 mmol) was dissolved in 5 ml of phosphate buffer (0.1 M) at pH 7.0. 1-Benzyl-3-carbamoyl-1,4-dihydropyridine (0.7 mmol) was added to this solution and the contents brought to 40 °C. Methanol was added dropwise to effect complete dissolution of the reductant and the contents incubated at this temperature for 24 h. The solution was evaporated to 5 ml, made basic to litmus and the filtrate run through a Sephadex G-10 column using water as eluant. The ninhydrin-active fraction was lyophilized, the resulting residue dissolved in D_2O in phosphate buffer (pH 7.0) and the NMR spectrum recorded. The spectrum was identical with that obtained for a mixture of 1-benzyl-3-carbamoylpyridinium chloride and pipecolic acid under identical experimental conditions.

One of the products of the glutamate dehydrogenase-catalyzed reaction between NADPH and PPA was identified as pipecolic acid by NMR. To a D_2O solution of 0.1 M phosphate at pH 8.9 containing PPA (70 mM) and NADPH (70 mM) was added lyophilized L-glutamate dehydrogenase powder (40 μM) (Sigma) and the progress of the reaction was followed by NMR from the appearance of pipecolic acid γ-CH_2 proton absorption (3.5 ppm). When the reaction was carried out under identical conditions without the enzyme, no visible reaction was noticed during the same period.

The enzyme-catalyzed reduction of $NADP^+$ by pipecolic acid was found to produce NADPH as one of the products by a spectrophotometric method. To a solution of L-pipecolic acid (1.0 M) in 0.1 M phosphate at pH 8.9 was added $NADP^+$ (1 mM) and glutamate dehydrogenase (0.25 mg/ml). A time-dependent increase in the absorbance at 340 nm was observed. There was a rapid depletion of this absorbance on the addition of α-ketoglutarate (0.1 mM) and ammonium sulfate (20 mM) to the reaction mixture confirming that NADPH is indeed one of the products of the reaction. The same reaction, when carried out in the absence of glutamate dehydrogenase, failed to produce any detectable amount of NADPH even after a much longer reaction time.

2.4. Kinetic procedure

Kinetic runs were followed in a Hewlett-Packard 8450A UV/Vis spectrophotometer as described previously [7]. The solutions in the cuvettes were kept stirred at all times and their temperature was maintained at 25.0 ± 0.1 °C. The reactions were initiated by adding to 2 ml of the thermostated solution 20–100 μl of the enzyme stock solution. The pH of the solutions measured before and after rate measurements remained constant.

2.5. Determination of pK_a values

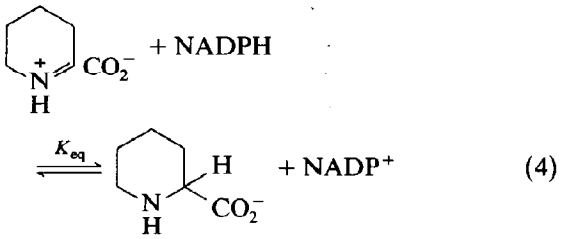
The acid ionization constants for PPA and pipecolic acid were determined using a standard potentiometric procedure [15]. The procedure involves titrating a solution of the compound (~ 0.01 M) against a concentrated solution of HCl or KOH and recording the pH. Corrections were made for the slight dilution involved in this process [15]. The pK_a value was determined from the inflection point of the sigmoidal curve. The acid ionization constants for PPA and pipecolic acid

were found to be 8.10 ± 0.03 and 10.72 ± 0.03, respectively, at $25.0 \pm 0.1°$C.

The pK_a values were assumed to be insensitive to the small changes in the ionic strength accompanying the titrations. The activity coefficients of the species involved in the equilibrium were assumed to be unity, since the concentrations of the substrate were low (~ 0.01 M).

2.6. Determination of the equilibrium constant for the PPA-pipecolic acid anion interconversion

We have measured the equilibrium constant, K_{eq}, for the model reaction (eqs 4 and 5).



(4)

$$K_{eq} = \frac{[\text{NADP}^+][\text{pipecolic acid anion}]}{[\text{NADPH}][\text{PPA}]} \qquad (5)$$

This was accomplished by observing the rate of NADPH production or depletion at different pH values at fixed amounts of PPA, L-pipecolic acid, glutamate dehydrogenase, $NADP^+$ and NADPH in 0.10 M phosphate-0.10 M Tris buffer. Under the experimental conditions, as the pH of the solution is gradually increased from pH 8.9, the rate of NADPH disappearance gradually diminishes to a point where the reaction reverses its direction, causing NADPH to be produced (fig. 1). The value of pH_{eq}, that pH at which the absorbance at 340 nm is invariant with time, was determined by interpolation. The value of K_{eq} was calculated from the pH_{eq} value. The concentration of free NADPH in solution was calculated by assuming that the enzyme is fully saturated with NADPH under the experimental conditions; E-NADPH is a tight complex at these pH values [16]. The concentrations of the PPA and pipecolic acid anions at pH_{eq} were calculated from a knowledge of their analytical concentrations and pK_a values (8.10 for PPA and 10.72 for pipecolic acid). We have determined the K_{eq} value over a range of pH_{eq} (9.0–9.2) by varying the analytical concentrations of the reactants and products, and found it to be a constant. An average value of 175 ± 30 was obtained for K_{eq}.

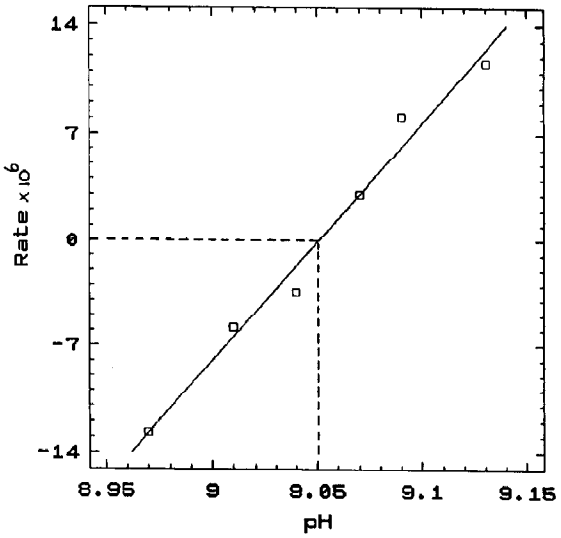


Fig. 1. Rate of appearance or disappearance of NADPH (ΔA_{340}/s) as a function of pH. [E] = 1.03 mg/ml; [L-pipecolic acid] = 1.20 M; [PPA] = 35 mM; [NADPH] = 145 μM; [$NADP^+$] = 2.95 mM; [Tris] = 0.1 M; [PO_4^{3-}] = 0.1 M. The pH, 9.05, at which the reaction is at equilibrium is determined by interpolation (dashed line).

2.7. Determination of $\Delta H°$ values

The standard enthalpies of ionization of PPA and pipecolic acid were determined to be 9.51 and 10.72 kcal/mol, respectively, by obtaining the pK_a values at different temperatures and then fitting the data to eq. 6.

$$\log K = -(\Delta H°/2.3RT) + C \qquad (6)$$

The value of $\Delta H°$ for the PPA-pipecolic acid anion reaction was determined by measuring pH_{eq} at various temperatures. The concentrations of PPA and pipecolic acid anion at pH_{eq} for a given temperature were calculated from the analytical concentration of these acids and their pK_a and $\Delta H°$ values of proton ionization; the concentrations of the coenzymes were determined by assum-

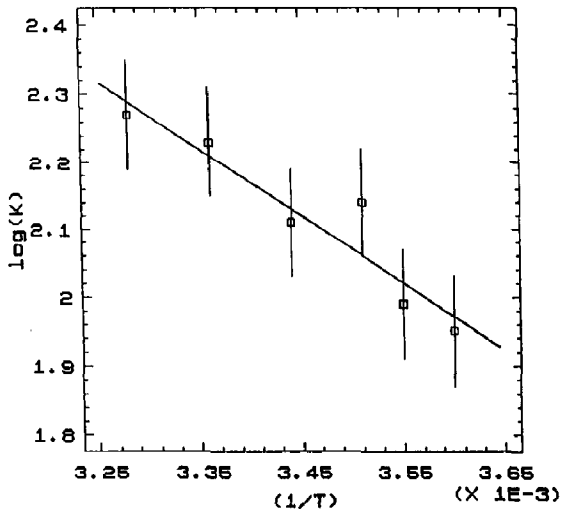


Fig. 2. Temperature dependence of the equilibrium constant. The ΔH° value for the reaction is determined from the slope (eq. 6) to be 5 ± 1 kcl/mol.

ing that the enzyme is fully saturated with only NADPH under the experimental conditions [5,16]. To correct for changes in the buffer pK values with temperature, the pH values of the reaction mixtures were measured at the reaction temperature. The dependence of K_{eq} on temperature is shown in fig. 2.

2.8. Reaction conditions

Measurements were made in 0.1 M Tris/0.10 M phosphate buffer. The solutions were adjusted to the desired pH values with hydrochloric acid or potassium hydroxide. Thus, for a given experiment all reaction mixtures had identical buffer compositions but different ionic strengths.

3. Results and discussion

We have found that the reduction of PPA by 1,4-dihydropyridines produces pipecolic acid and the corresponding pyridinium ions and that glutamate dehydrogenase catalyzes this reaction (fig. 3). The nonenzymatic reaction in the other direction, however, does not proceed at a measurable speed under the experimental conditions, which cover a pH range of 8.0–9.5. On the other hand, the PPA-pipecolic acid reaction is freely reversible in the presence of a catalytic amount of glutamate dehydrogenase when L-pipecolic acid and $NADP^+$ (or NAD^+) are used as reactants. We have noted that the enzymatic oxidation of DL-pipecolic acid occurs at half the rate of the L isomer indicating that only the L isomer is a substrate for the enzyme; the D isomer is neither a substrate nor an inhibitor for the enzyme.

The equilibrium constant, K_{eq}, for the reaction (eq. 5) has been found to be 175 ± 30 corresponding to a ΔG° value of -3.1 ± 0.1 kcal/mol. This value is not significantly different from the ΔG° value of -3.3 ± 0.1 kcal/mol, obtained for the reduction of Δ^1-pyrroline 2-carboxylic acid by NADPH to yield proline anion and $NADP^+$ [6]. The observation that the reduction of the iminium ion is only modestly exergonic will be important in the interpretation of various kinetic data of the reaction including the primary kinetic deuterium isotope effect with 4,4-dideuterated NADH, tun-

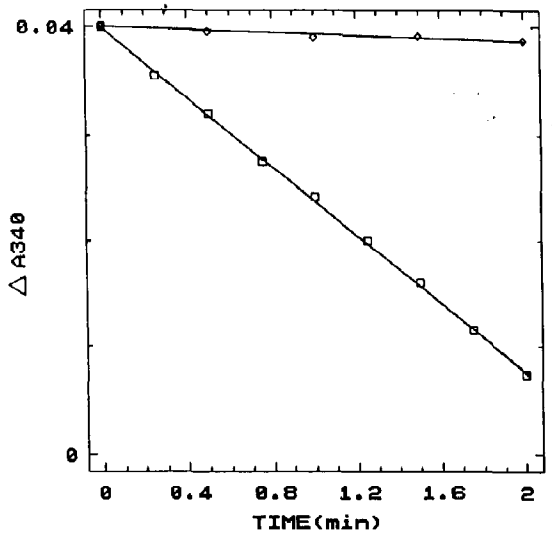


Fig. 3. Time dependence of NADPH disappearance for the nonenzymatic (□) and the enzyme-catalyzed (■) reactions. [E] = 1.23 mg/ml; pH = 7.63; [NADPH] = 150 μM; [PPA] = 12 mM; $T = 25.0\,^\circ$C; [Tris] = 0.1 M; $[PO_4^{3-}] = 0.1$ M. The observed rate with the enzyme (0.0162 A/min) is corrected for the nonenzymatic rate (6.6×10^{-4} A/min) to obtain the corrected enzyme-catalyzed rate (0.0155 A/min).

neling by hydride ion and the Brönsted exponent with substituted dihydropyridines.

It has become apparent that free energies alone do not provide sufficient information to characterize the steps in chemical and biochemical processes [17]. Consequently, we have determined the ΔH° and ΔS° values for the reaction from the temperature dependence of K_{eq} values; the concentration of PPA and L-pipecolic acid anion needed for the calculation of K_{eq} at various temperatures was calculated from their pK_a values (8.10 and 10.72 for PPA and pipecolic acid, respectively, at 25 °C) and the ΔH° values for proton ionization of 9.51 kcal/mol for PPA and 10.72 kcal/mol for pipecolic acid.

The equilibrium reduction of PPA to give pipecolic acid anion (eq. 4) has a ΔH° of 5 ± 1 kcal/mol and a ΔS° of 27 ± 4 e.u. A large value for ΔS° may arise from a change in the number of product molecules being produced from reactant molecules and/or a significant change in the solvation characteristics of reactants relative to products. Since there is no change in the number of reactant and product molecules we assign the large positive ΔS° value to a considerable amount of solvent organization in the reactants relative to the products. It is not clear from the structures of reactants and products why several solvating water molecules are virtually freed upon formation of the products. In any case, the data suggest that the solvent reorganization must be an important factor contributing toward the free energy of activation for the oxidation of pipecolic acid anion by $NADPH^+$.

The thermodynamic parameters ($K_{eq} = 175 \pm 30$, $\Delta G^\circ = -3.1 \pm 0.1$ kcal/mol, $\Delta H^\circ = 5 \pm 1$ kcal/mol and $\Delta S^\circ = 27 \pm 4$ e.u.) pertain to the equilibrium shown in eq. 4. In this equation, the imino acid and the amino acid are represented in terms of the active species undergoing the redox reaction. However, the equilibrium parameters for the reaction in eq. 3 which represents the amino acid and imino acid as major species (at pH 6.0, for example), can easily be calculated by combining the thermodynamic data for the reaction in eq. 4 with those for the proton ionization of pipecolic acid ($K_a = 1.92 \times 10^{-11}$ M; $\Delta G^\circ = 14.6$ kcal/mol, $\Delta H^\circ = 10.7$ kcal/mol and $\Delta S^\circ = -13.1$ e.u.). The thermodynamic constants corresponding to eq. 3 are $K_{eq} = (1.1 \pm 0.2) \times 10^{-13}$ M, $\Delta G^\circ = 17.7 \pm 0.6$ kcal/mol, $\Delta H^\circ = 6 \pm 1$ kcal/mol and $\Delta S^\circ = -40 \pm 6$ e.u.

What are the implications of the thermodynamic constants on the glutamate dehydrogenase catalyzed glutamate oxidation? To answer this question, we will assume that the thermodynamic constants for eq. 4 are not significantly different for the redox step of the glutamate reaction (glutamate–α-iminoglutarate interconversion) and apply the observed ΔH° value of -5 kcal/mol, obtained for the oxidation of pipecolic acid anion, to the redox step of the glutamate reaction; it is likely that glutamate binds and reacts with the enzyme-$NADP^+$ complex with its amino group unprotonated [6]. Colen et al. [18] studied the temperature dependence of the initial transient velocities associated with the burst of NADPH production which results upon mixing stoichiometric amounts of glutamate dehydrogenase with L-glutamate and oxidized coenzyme. They found that this transient phase is characterized by a large negative heat capacity of activation and attributed this observation to the presence of hidden equilibria comprising the transient phase. Although the hydride transfer step is a part of the transient phase, its ΔH° value of about -5 kcal/mol is too modest to produce a large negative heat capacity of activation; such heat capacities can be produced by a hidden equilibrium only if it has a large ΔH° value (of either sign). We conclude that the burst phase of the glutamate reaction gives rise to a heat capacity of activation due mainly to the presence of a nonredox step of a large ΔH° value.

The purpose of this investigation is to provide a set of thermodynamic parameters characterizing the reduction of an iminium ion so that the catalysis of glutamate oxidation by glutamate dehydrogenase may be understood in thermodynamic terms. While we have determined the thermodynamic parameters, it must be recognized that the particular reaction investigated utilizes only a portion of the catalytic machinery of the enzyme. Also, we have used a stable cyclic imine to model the unstable acyclic intermediate, α-iminoglutarate. While these limitations apply to the extrapolation of the results from the model reaction to the

glutamate reaction, the thermodynamic constants for the PPA reaction are free of such limitations when they are applied to the analysis of the energetics and mechanism of the same reaction in the nonenzymatic and enzyme-catalyzed pathways.

Acknowledgements

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References

1 J.S. Blanchard and W.W. Cleland, Biochemistry 19 (1980) 3543.
2 A. di Franco, Eur. J. Biochem, 45 (1974) 407; E. Silverstein and G. Sulebele, Biochemistry 12 (1973) 2164.
3 M. Iwatsubo and D. Pantaloni, Bull. Soc. Chim. Biol. 49 (1967) 1563.
4 J.M. Jallon, Y. Risler and M. Iwatsubo, Biochem. Biophys. Res. Commun. 67 (1975) 1527.
5 J.E. Rife and W.W. Cleland, Biochemistry 19 (1980) 2321.
6 R. Srinivasan and H.F. Fisher, Biochemistry 24 (1985) 618.
7 R. Srinivasan, R.T. Medary, H.F. Fisher, D.J. Norris and R. Stewart, J. Am. Chem. Soc. 104 (1982) 807.
8 D. Mauzerall and F.H. Westheimer, J. Am. Chem. Soc. 77 (1955) 2261.
9 M.A. Roseman, Ph.D. Thesis, Michigan State University, East Lansing (1971).
10 A. Meister, J. Biol. Chem. 206 (1954) 577.
11 D.G. Dross and H.F. Fisher, J. Biol. Chem. 245 (1970) 2612.
12 J.A. Olson and C.B. Anfinsen, J. Biol. Chem. 197 (1952) 67.
13 R.B. McComb, L.W. Bond, R.W. Burnett, R.C. Keech and G.N. Bowers, Jr, Clin. Chem. 22 (1976) 141.
14 J.M. Siegel, G.A. Montgomery and R.M. Bock, Arch. Biochem. Biophys. 82 (1959) 288.
15 A. Albert and E.P. Serjeant, Ionization constants of acids and bases (Metheun, London, 1962) ch. 2.
16 H.F. Fisher, S. Maniscalco, C. Wolfe and R. Srinivasan, Biochemistry 25 (1986) 2910.
17 R.L. Biltonen and E. Freire, CRC Crit. Rev. Biochem. 5 (1978) 85.
18 A.H. Colen, R.T. Medary and H.F. Fisher, Biopolymers 20 (1981) 879.