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PHYSICOCHEMICAL AND KINETIC PROPERTIES OF BEEF LIVER ARGININOSUCCINASE

STUDIES IN THE PRESENCE AND ABSENCE OF ARGINASE

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Summary

Physicochemical properties of beef liver argininosuccinase (EC 4.3.2.1) are reported, which show that the operational kinetic unit at pH 7.5 in the concentration range used for enzymic assays is of molecular weight 203 000. This information is utilized in the interpretation of initial velocity studies of the forward reaction (argininosuccinate as initial substrate) where, in an Eadie-Hofstee plot, deviation from linearity is observed. These results are examined with the aid of a rate equation, formulated as a ratio of two polynomials in initial substrate concentration, which provides a quantitative description of the system in terms of cooperative effects. The kinetics of the reverse reaction catalysed by argininosuccinase were also investigated by performing experiments of different design in which the rate of change of fumarate concentration was monitored as a function of time. Comparison of these results with numerical solutions obtained by integrating the differential rate equation reflecting the reversible reaction permitted estimation of the relevant kinetic parameters. One of the latter experiments involved a coupled assay with argininosuccinase and arginase as the consecutive catalysts and indicated that no chemical interaction occurred between the enzymes. This observation, which is relevant to consideration of fluxes in the urea cycle, was supported by both sedimentation velocity studies on mixtures of the enzymes and by other kinetic analysis.

Introduction

Argininosuccinate (L-argininosuccinate arginine lyase, EC 4.3.2.1) which is one of the four enzymes of the ornithine-urea cycle catalyses the reversible

conversion of argininosuccinate to fumarate and arginine [1,2]. The method of purification and initial physicochemical characterization of the enzyme have been reported previously [2—6]. This paper extends these observations by paying particular attention to the state of aggregation of the enzyme in the concentration range encountered in enzymic assays, to the kinetic behaviour in initial velocity studies conducted with low substrate concentrations, and to kinetic parameters describing the time course of the reaction as it approaches its equilibrium state. The possibility is also explored that argininosuccinase may interact with arginase (L-arginine amidino hydrolase, EC 3.5.3.1), an adjacent enzyme in the urea cycle. Such an interaction, which has been suggested for other enzymes of arginine metabolism in yeast and fungi [7,8], would complicate the interpretation of coupled assay results [9—11] and affect fluxes associated with the urea cycle.

Experimental

Preparation and physicochemical properties of argininosuccinase

The purification of argininosuccinase from 3-kg batches of young beef liver proceeded by the method outlined by Ratner [5], with the modification that in Steps 5 and 6 chromatography was effected using single larger columns (5.6 × 35 cm, CM-cellulose and 4 × 29 cm, DEAE-cellulose). The final crystallization [5] gave 65 mg of protein, with specific activity 1400 Ratner units · mg^{-1} [5], corresponding to a k_{cat} of 4.2 · 10^3 min^{-1} at 38°C, and to a 24% yield.

Three criteria were used to establish the homogeneity of the enzyme preparation so obtained. First, a single band was observed at 20°C both in polyacrylamide gel electrophoresis conducted by the method of Davis [12] (7.5% acrylamide, pH 8.3, 0.006 M Tris/0.037 M glycine electrode buffer) and in electrophoresis on cellulose acetate using a Beckman R100 Microzone apparatus. Secondly, in a sedimentation velocity experiment conducted at 20°C with a 0.3 g · dl^{-1} solution in 0.05 M Tris · HCl, 0.05 M NaCl buffer, pH 7.5, using a Spinco model E ultracentrifuge, a single sedimenting schlieren peak with $s_{20,w}$ of 9.4 S was observed. This value is in agreement with that of 9.3 S previously reported [4]. Moreover analysis of the shape of the sedimenting boundary by the method described by Baldwin [13] confirmed the essential homogeneity of the sample with respect to hydrodynamic parameters. Similar results were obtained when sedimentation velocity analysis was conducted at 20°C in 0.066 M phosphate buffer, pH 7.5. Thirdly, a sedimentation equilibrium experiment of the meniscus-depletion type [14] also conducted at pH 7.5 (0.066 M phosphate buffer) and 20°C yielded a linear plot of log fringe displacement versus radial distance squared: the experiment, performed with an initial loading concentration of 0.04 g · dl^{-1} and at an angular velocity of 15 000 rev./min, therefore demonstrates homogeneity with respect to molecular weight. The final concentration range encompassed was 0.008—0.132 g · dl^{-1} and the indicated molecular weight was 203 000, which compares favourably with the previously reported value [3] of 202 000.

It is noted that the lowest measurable concentration encompassed by the sedimentation equilibrium experiment was 0.008 g · dl^{-1} corresponding to a

molar concentration of $3.94 \cdot 10^{-7}$ M which is approximately 100 times that employed in enzyme kinetic studies. In order to examine the effect of dilution on the state of aggregation of the enzyme, two frontal analysis experiments [15,16] were performed on a Sephadex G-200 column (1.27 × 50 cm) employing plateau concentrations of $4.92 \cdot 10^{-7}$ and $4.92 \cdot 10^{-9}$ M in the same pH 7.5 phosphate buffer at 20°C. From the observed weight average elution volumes (corresponding to the median bisector of the advancing front and monitored by enzymic assay) it was possible to calculate K_{av} [17] using the void volume of the column found with Blue Dextran 2000. For both plateau concentrations the value of K_{av} was 0.21 ± 0.1 corresponding to a molecular weight of approx. 200 000 [17]. This set of experiments correlates in concentration range with the sedimentation equilibrium experiment and shows that the operational unit at high dilution continues to be of molecular weight 203 000.

Kinetic experiments with argininosuccinase alone

In the study of the forward reaction with argininosuccinate as initial substrate, initial velocities were determined by measuring the increase of fumarate concentration with time spectrophotometrically [5] at 240 nm with a Carey 14 spectrophotometer (slit setting 0.08 mm). Control experiments were performed to ensure a linear concentration versus absorbance plot. The detailed protocol of a typical experiment was as follows: 2 ml of 0.066 M phosphate buffer, pH 7.5, was introduced into a 1 cm quartz cuvette thermostated at 25°C followed by 0.25-ml aliquots of enzyme and substrate solutions, pre-equilibrated at 25°C in the same buffer. After rapid mixing, recording was commenced within 10—15 s of the initiation of the reaction, with the recorder chart speed set to yield an initial slope of approx. 45° on the tracing. Initial velocities were determined as the average values obtained from experiments performed in triplicate. In these experiments, argininosuccinate used as initial substrate was prepared and crystallized by the method described by Ratner [18]. In determining the initial substrate concentrations, account was taken of the observation [19,20] that in solution and on storage argininosuccinate cyclizes to five and six membered ring anhydrides which are neither substrates nor inhibitors of argininosuccinase. Thus, selected assays were allowed to proceed to equilibrium and the equilibrium concentration of fumarate, [F], was determined spectrophotometrically, and used in the following relation,

$$[\text{argininosuccinate}]_{\text{initial}} = ([F]^2/K_{eq}) + [F] \quad (1)$$

where K_{eq} ($3.22 \cdot 10^{-3}$ M) was determined in a separate experiment in which a solution initially containing fumarate and arginine of known concentration was allowed to reach equilibrium. In practice the correction was small, since cyclization proceeded to the extent of only approx. 2% when a solution of argininosuccinate was stored for a period of 7 days at 4°C.

In studying the kinetics of the reverse reaction catalysed by argininosuccinase, with fumarate and arginine as initial substrates, the experimental procedure was as described above, with the time-dependent depletion of fumarate being recorded spectrophotometrically. The kinetics of both forward and reverse reactions were studied in 0.066 M phosphate, pH 7.5, the environment used for the physicochemical characterization of the enzyme, and also in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5.

The coupled reaction with arginase

Certain experiments were performed in the joint presence of argininosuccinase and arginase, the latter being obtained from beef liver by the method of Harell and Sokolovsky [21] as described previously (Kuchel, P.W., Nichol, L.W. and Jeffrey, P.D., unpublished). The arginase was stored in the presence of 0.01 M $MnCl_2$, since Mn^{2+} are required for structural stability [22]. It was required, however, to remove excess Mn(II) prior to coupled assay experiments since these metal ions were found to be a strong inhibitor of argininosuccinase. The removal was effected by a 1.5 h rapid dialysis technique [23] which resulted in only an approx. 5% loss of arginase activity and a sample, 95% of which migrated in sedimentation velocity experiments with the sedimentation coefficient of 6.1 S characteristic of intact arginase (ref. 21 and Kuchel, P.W., Nichol, L.W. and Jeffrey, P.D., unpublished). The coupled assay experiments were of two kinds. First, consecutive reactions were studied in which argininosuccinate as initial substrate was converted to fumarate and arginine, the latter compound being hydrolysed to form ornithine and urea. This sequence of reactions was conducted in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5, at 25°C by monitoring urea production by the diacetyl monoxime method (ref. 24 and Kuchel, P.W., Nichol, L.W. and Jeffrey, P.D., unpublished). Secondly, arginine and fumarate were used as initial substrates in the cited Tris buffer, a mixed solution of argininosuccinase and freshly pre-dialysed arginase being added. In this reaction mixture arginine forms ornithine and urea under the influence of arginase, and argininosuccinate under the influence of argininosuccinase. This joint removal of arginine will be shown to be reflected in the change of fumarate concentration with time which was monitored spectrophotometrically as described above.

Results

Initial velocity studies on the forward reaction

Fig. 1 presents results obtained employing argininosuccinate as the initial substrate (concentration range of $1.01 \cdot 10^{-5}$ M to $2.02 \cdot 10^{-3}$ M) in reaction mixtures (0.066 M phosphate buffer, pH 7.5) in which the sole enzyme present was argininosuccinase. Entirely similar results were found when different enzyme preparations were used and when the buffer was 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5. Previous studies of a similar kind [2] detected the deviation from linearity which is apparent in the region of high v_o (high initial substrate concentration) and attributed it to a negative cooperative effect [2,25]. In this previous analysis, the slope of the linear segment in the range of v_o $1 \cdot 10^{-5}$ to $2 \cdot 10^{-5}$ M · min^{-1} was used to determine an apparent K_m of approx, $5 \cdot 10^{-5}$ M, a value in accord with the present results. The broken line in Fig. 1 was calculated theoretically on the basis of a model (to be discussed in detail later) which invokes only negative cooperativity between the catalytic sites. It is a reasonable representation of the experimental results when account is taken of experimental scatter, particularly important in relation to the points obtained at low substrate concentrations, a range not previously examined. It cannot be assumed, however, that the model involving only negative cooperative effects uniquely describes the results. This is emphasized by the solid line

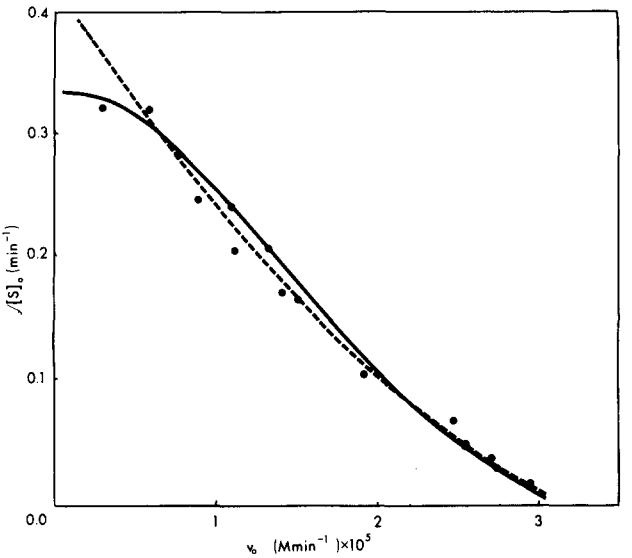

Fig. 1. Results obtained in the study of the initial velocity of the reaction catalysed by beef liver argininosuccinase with argininosuccinate as initial substrate in 0.066 M phosphate buffer, pH 7.5, 25°C. The results are plotted in the Eadie-Hofstee format with the initial velocity v_o as the abscissa and $v_o/[S]_o$ as the ordinate where $[S]_o$ is the initial substrate concentration. Experimental points are denoted by (•). The broken and solid lines were computed on the basis of Eqn 3 utilizing the parameters reported in the text. - - - - - -, negative cooperative effects alone; ———, positive and negative cooperative effects.

in Fig. 1 which was calculated theoretically on a different basis to be given later.

Studies on the reverse reaction

Since the reaction, fumarate + arginine ⇌ argininosuccinate, does not involve water and includes only those reactants given in the equation it may reasonably be classified, in relation to the enzyme argininosuccinase, as a Bi Uni mechanism. Moreover, as arginine was found in this work and in previous studies [2] to be a pure competitive inhibitor for the forward reaction (initial substrate argininosuccinate) with apparent inhibition constant $1.6 \cdot 10^{-3}$ M, it is entirely reasonable to explore the postulate that the reaction is of the ordered sequential type, denoted by,

where A denotes arginine, F fumarate and AS argininosuccinate. On the basis that the deviation from linearity, seen in Fig. 1, may be neglected in an initial analysis of the results, the appropriate steady-state rate equation reflecting the operation of both forward and reverse reactions may be written [26], as,

$$v = \frac{V_f V_r \left([AS] - \frac{[F][A]}{K_{eq}}\right)}{K_{AS} V_r + V_r[AS] + \frac{K_A V_f[F]}{K_{eq}} + \frac{K_F V_f[A]}{K_{eq}} + \frac{V_f[F][A]}{K_{eq}} + \frac{V_r[AS][F]}{K_F^i}} \qquad (2)$$

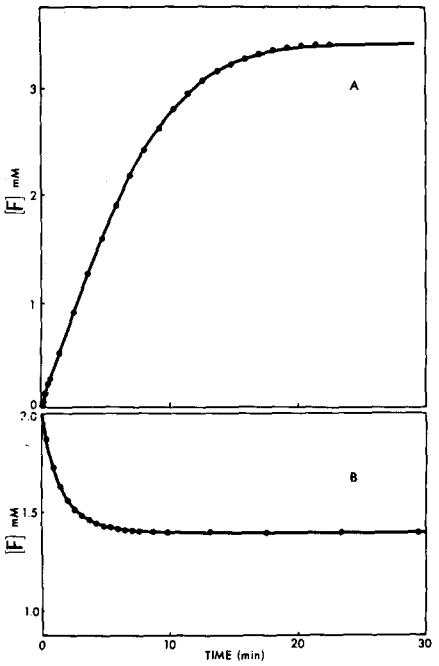

Fig. 2. Experimental points (•) of the concentration of fumarate, [F], as a function of time obtained in experiments conducted with beef liver argininosuccinase as the enzyme in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5, at 25° C. The solid line in each case was found by numerical integration of Eqn 2, using the second-order predictor-corrector method, and the following values of parameters defined in the text: $K_{eq} = 3.22 \cdot 10^{-3}$ M, $K_{AS} = 5 \cdot 10^{-5}$ M, $K_A = K_F = 1 \cdot 10^{-4}$ M, and $K_A^i = 1.0 \cdot 10^{-3}$ M. (A) Argininosuccinate ($3.74 \cdot 10^{-4}$ M) was used as initial substrate, the fumarate production being monitored spectrophotometrically. For this experiment $V_f = 4.4 \cdot 10^{-4}$ M · min^{-1} was taken to equal V_r in the numerical simulation. (B) Fumarate and arginine each of concentration 2 mM were used as initial substrates, the depletion of fumarate being followed spectrophotometrically. $V_f = V_r = 5 \cdot 10^{-4}$ M · min^{-1}.

where v is the velocity of the reaction at any time t; V_f and K_{AS} are the maximal velocity and Michaelis constant found by employing argininosuccinate as initial substrate; V_r and K_A are the corresponding parameters for studies in which F is in excess and A is varied as initial substrate; V_r and K_F the corresponding parameters when A is held in excess; and K_F^i is the non-competitive inhibition constant for F on the reaction initiated with AS. A test of the proposed model and implicit assumptions may be made by comparing experimental results obtained as a function of time with theoretical solutions found by integrating numerically Eqn 2.

The solid points in Fig. 2A were obtained experimentally by monitoring the increase of fumarate concentration with time in an experiment where argininosuccinate ($3.74 \cdot 10^{-4}$ M) was used as the sole initial substrate. The solid line is the numerical solution of Eqn 2 obtained by employing the set of parameters reported in the caption to Fig. 2. The selection of these parameters was aided by the following considerations. Of the seven required parameters, the values of K_{eq} ($3.22 \cdot 10^{-3}$ M), K_{AS} ($5 \cdot 10^{-5}$ M) and V_f ($4.4 \cdot 10^{-4}$ M · min^{-1}) are known. Moreover, on the basis of observations made by Ratner et al. [1], $V_f = V_r$ and $K_A = K_F$. It remains therefore to assign a value to K_F which

may be done on the basis that $K_F = K_{eq}\ K_{AS}\ V_r/V_f\ K_A^i$ [26] where K_A^i is the competitive inhibition constant of arginine with argininosuccinate as initial substrate. The value of K_A^i was searched using the previously reported apparent value of $1.6 \cdot 10^{-3}$ M as the starting value to obtain the solid line shown in Fig. 2A, which is seen to fit well the experimental points.

A further test of the proposed mechanism was made by performing an experiment in which fumarate and arginine each of concentration $2 \cdot 10^{-3}$ M were used as initial substrates and the depletion of fumarate with time was recorded spectrophotometrically. The results are shown as the solid points in Fig. 2B where again it is seen that the numerical solution of Eqn 2 fits the results. This simulation employed the same values of kinetic parameters as were used in Fig. 2A except that V_f was assigned the appropriate value for the different concentration of argininosuccinase employed.

An investigation of the possible interaction of argininosuccinase and arginase

The first step in this investigation was to study mixtures of the two enzymes in the absence of substrates. A typical experiment involved the construction of a solution containing 0.25 g · dl^{-1} of argininosuccinase and 0.30 g · dl^{-1} of arginase (pre-dialysed free of excess manganese) in pH 7.5 phosphate buffer and subjecting this solution to sedimentation velocity. The result is shown in the lower pattern of Fig. 3. The upper pattern is a control experiment performed with the same concentration of arginase alone in a separate cell with a wedge window. The sedimentation coefficient of arginase alone proved to be identical with that of the slower sedimenting peak in the mixture, which by area determination corresponded to a concentration 3% greater than that of the arginase concentration initially introduced. This difference is in quantitative agreement with the Johnston-Ogston equation [27] and shows that, while slight frictional interactions occur between proteins in the mixture on migration, no chemical interaction operates. Entirely similar results were found in the following environments: 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5, 20°C and 0.02 M maleate buffer, pH 6.0, 20°C. The latter buffer was selected since pH 6.0 is intermediate between the isoelectric points of arginase (pH 5.9) and argininosuccinase (pH 6.1) found by isoelectric focusing experiments as described previously (Kuchel, P.W., Nichol, L.W. and Jeffrey, P.D., unpublished).

The above sedimentation velocity results do not exclude the possibility of a heterogeneous association between the enzymes mediated by one or more of the various components of reaction mixtures encountered in a coupled assay. Experiments were performed with argininosuccinate as initial substrate, in the presence of both enzymes: in these experiments the rate of production of urea was monitored and thus, for the first time, the coupled sequence of reactions catalysed by both enzymes was being studied in the presence of all relevant ligands. With the large excess of arginase used (molar ratios of enzymes, 10 : 1) the rate of urea production equalled the rate of argininosuccinate disappearance [28,29]. Accordingly, such studies performed with initial argininosuccinate concentration in the range 0.1—3 mM yielded the initial velocity parameters V_f ($1.3 \pm 0.2 \cdot 10^{-5}$ M · min^{-1}) and K_{AS} ($1.0 \pm 0.2 \cdot 10^{-4}$ M) pertinent to Eqn 2. The value of V_f compares favourably with that of $1.2 \pm 0.1 \cdot 10^{-5}$ M · min^{-1} found with the same sample of argininosuccinase studied alone, while

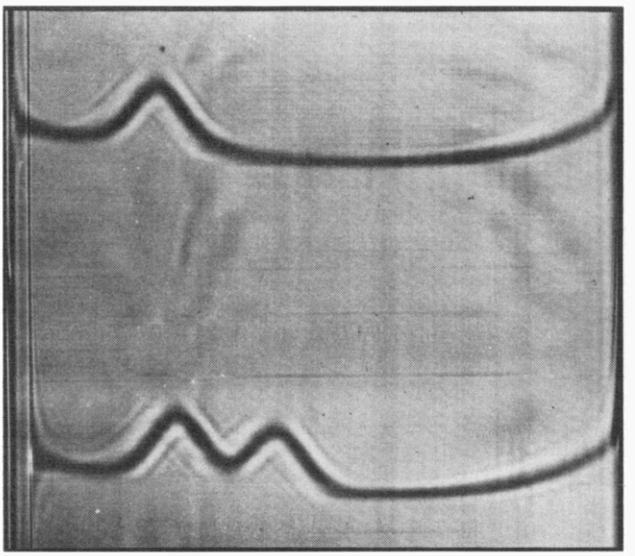

Fig. 3. Sedimentation velocity patterns obtained at an angular velocity of 60 000 rev./min with a 0.30 g · dl^{-1} solution of arginase (upper pattern) and a mixture of 0.30 g · dl^{-1} arginase and 0.25 g · dl^{-1} argininosuccinase (lower pattern) in phosphate buffer, pH 7.5.

that of $K_{A\,S}$ is also in agreement with the result shown in Fig. 1 when account is taken of the relatively high initial substrate concentration range employed (a region where the slope of the curve and hence the apparent Michaelis constant increases from the value of $5 \cdot 10^{-5}$ M with increasing substrate concentration). The lack of interaction between the enzymes suggested by these results was confirmed by initial velocity studies which show that no inhibition of argininosuccinase by arginase (or vice versa) arose when the rate of production of fumarate (or urea) was monitored.

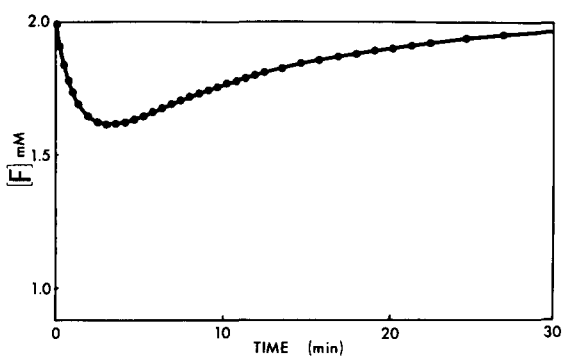

Fig. 4. Experimental points (•) showing the change of fumarate concentration, [F], with time in an experiment where argininosuccinase and arginase were both present with initial substrates arginine and fumarate each of concentration 2 mM in 0.05 M Tris · HCl, 0.05 M NaCl, pH 7.5, 25° C. The solid line was computed on the basis of Eqn 2 with the same values for the parameters as reported in the caption to Fig. 2B, combined with an equation describing the depletion of arginine by arginase ($K_m = 5.0 \cdot 10^{-3}$ M, $V = 1.5 \cdot 10^{-3}$M · min^{-1}), account being taken of the competitive inhibition of arginase by ornithine ($K_I = 3.0 \cdot 10^{-3}$ M).

Finally, in this connection an experiment was performed in which to a mixture of argininosuccinase and arginase was added a solution of fumarate and arginine each of final concentration 2 mM. The experimental points obtained are shown in Fig. 4. The observed change of fumarate concentration with time is due to the combined effects of the reversible reaction encompassed by Eqn 2 and the depletion of arginine by arginase described by the end-product competitive inhibition Michaelis-Menten equation which considers the competitive inhibition of arginase by ornithine. The solid line was computed by integrating numerically these combined equations, and utilizing the basic kinetic parameters previously reported. As with Fig. 2, the fit of the theoretical simulation to the experimental points shown in Fig. 4 may be judged satisfactory, particularly in relation to the experimentally observed minimum. The result therefore supports not only the basic mechanism invoked and the kinetic parameters employed; but also provides further evidence for lack of interaction between arginase and argininosuccinase.

Discussion

A direct interpretation of the available physicochemical evidence (both sedimentation equilibrium and frontal chromatography) is that argininosuccinase exists as a single species of molecular weight 203 000 at the concentrations used in enzymic assaysThe results in Fig. 1, therefore, require discussion in terms of this unit, which is known to possess four catalytic sites [2,6]. The basic equation of Adair [30] for a four-site model may be written in kinetic format [31] as,

$$v_0 = \frac{V}{4}\left\{\frac{\alpha_1 [S]_0 + \alpha_2 [S]_0^2 + \alpha_3 [S]_0^3 + \alpha_4 [S]_0^4}{1 + \alpha_1 [S]_0 + (\alpha_2/2) [S]_0^2 + (\alpha_3/3) [S]_0^3 + (\alpha_4/4) [S]_0^4}\right\} \qquad (3a)$$

where V is the maximal velocity ($3.2 \cdot 10^{-5}$ M $\cdot$ min^{-1}) and the coefficients are identified in terms of association equilibrium constants as,

$$\alpha_1 = 4K_1 \quad ; \quad \alpha_2 = 12K_1K_2$$
$$\alpha_3 = 12K_1K_2K_3 \; ; \quad \alpha_4 = 4K_1K_2K_3K_4 \qquad (3b)$$

In this formulation, the four catalytic sites on the enzyme have been taken as equivalent, whereupon the single intrinsic association constant K_1 suffices to describe the binding of one substrate molecule to any of the four sites. If the sites are also independent, then $K_1 = K_2 = K_3 = K_4$ and Eqn 3a becomes $v_o = V K_1 [S]_o/(1+K_1 [S]_o)$: in this case, the Eadie-Hofstee plot would be linear. Inequalities between the values of K result in deviations from linearity and may be interpreted in terms of cooperative effects between the equivalent catalytic sites rendering them dependent.

The broken line in Fig. 1 was computed using Eqn 3 with $K_1 = 1.3 \cdot 10^4$ M^{-1}, $K_2 = 1.1 \cdot 10^4$ M^{-1}, $K_3 = 0.9 \cdot 10^4$ M^{-1} and $K_4 = 0.6 \cdot 10^4$ M^{-1}. This interpretation of the results, therefore, suggests that the operation of negative cooperative effects proposed earlier [2,25] to explain the results found with high substrate concentrations suffices to describe the results over the whole

concentration range within experimental error. It is noted, however, that the same curve would arise by considering the four sites to be non-equivalent and independent but there is no evidence for this proposal. Moreover, it is not claimed that the parameters reported represent a set of values which uniquely describe the system. This is emphasized by the solid line in Fig. 1 which also reasonably describes the results and was calculated with Eqn 3 and $K_1 = 1.0 \cdot 10^4\ M^{-1}$, $K_2 = 1.5 \cdot 10^4\ M^{-1}$, $K_3 = 0.9 \cdot 10^4\ M^{-1}$ and $K_4 = 0.7 \cdot 10^4\ M^{-1}$. This set of values is consistent with the operation of both negative and positive cooperative effects, a joint operation originally discussed by Cook and Koshland [32].

The important point is, however, that for both interpretations $K_1 \approx K_2 \approx K_3 \approx K_4$, implying as is evident in Fig. 1 that the deviations from linearity are of small magnitude and are accordingly difficult to interpret unambiguously with available experimental precision. At the same time, this observation supports the use of the hyperbolic relation implicit in Eqn 2, and hence indicates that the kinetic parameters summarized in the caption to Fig. 2 may be taken as reasonable estimates of the constants characterizing the reversible reaction. Indeed, their use led to numerical simulations which closely fit experimental points (Figs 2A, 2B and 4) obtained in experiments of quite different design. These fits provide the additional information supported by sedimentation velocity analysis (Fig. 3) that arginase and argininosuccinase do not chemically interact in the environments studied, information which is directly relevant to the formulation of rate equations describing fluxes within the urea cycle.

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