APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 8, 135–144 (1983)

Comparison of the Coupling Recoveries of Immobilized Aspartate Aminotransferase

Specific Activity, Enzyme-Bound Coenzyme, and Transaminationable Active Centers

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Received August 11, 1982; Accepted December 13, 1982

Abstract

Aspartate aminotransferase (AspAT, EC 2.6.1.1)† was bound on CNBr-activated Sepharose and the effects of immobilization on the maximum velocity, biologically active pyridoxal- 5′-phosphate (PLP), and transaminationable active centers were studied. By comparing these parameters of soluble and immobilized enzyme the factors decreasing the observed reaction rate upon immobilization were evaluated.

Ninety percent of the soluble protein in the coupling mixture was bound to the support. The amount of enzyme-bound PLP of immobilized preparation was 83% of that of the soluble one. The coupling recovery of specific activity was 46%, which was 10%-units lower than that of the transaminationable active centers. This difference depends on the fact that a part of the active centers of immobilized enzyme had lower catalytic rate, due to the enzyme–matrix interactions or internal mass transfer limitations, than the others. The immobilized catalytically active AspAT had 80% of the turnover efficiency of the soluble enzyme. The affinity of the enzyme to its substrates did not significantly change upon immobilization, neither did the pH profile.

Index Entries: Immobilized aspartate aminotransferase; aspartate aminotransferase, immobilized pyridoxal-5′-phosphate; aminotransferase, immobilized aspartate.

†Abbreviations used: AspAT, aspartate aminotransferase, EC 2.6.1.1; PLP, pyridoxal-5′-phosphate; PMP, pyridoxamine 5′-phosphate; FMN, flavine mononucleotide; α-ktg, α-ketoglutarate; L-asp, L-aspartate; *s*, slope; *V*, maximum velocity; *v*, initial velocity.

0273-2289/83/0400-0135$02.00

Introduction

When an enzyme once has been immobilized on a solid support, the coupling results are generally expressed by bound protein and activity. However, immobilization affects both the intrinsic catalytic properties of the enzyme and the effective concentrations of substrates and products (*1, 2*). Therefore, the specific activity alone poorly illustrates the effects of immobilization on enzymes.

The diffusion barriers must be separated from the observed results before any comparison of the intrinsic properties of soluble and bound enzyme is possible, along with an evaluation of the effects of immobilization on enzyme structure, and the significance of enzyme–matrix interactions (*3*). Steady-state dependence of the reaction rate of one-substrate reactions on the diffusion effects has been presented by several investigators (*1, 2, 4–8*). Experimental verification of these theories requires knowledge of the diffusion parameters of the system and complicated kinetic determinations. Two-substrate reactions are still more complicated (*9–11*).

The amount of active enzyme in support has been estimated in several investigations with unkinetic active-site titrations (*3*). However, they usually produce only the maximum number of bound intact active sites, not necessarily the quantity of enzyme able to convert substrate to product. Many two-substrate enzymatic reactions undergo their half-reactions stoichiometrically, at least in the presence of large excess of the other substrate. Hence, the number of catalytically active enzyme sites can be measured, even though there still remains a possibility that the intrinsic catalytic rate of active centers is altered upon immobilization.

Aspartate aminotransferase (AspAT), a pyridoxal-5′-phosphate (PLP)-dependent enzyme, catalyzes the following reaction via a ping-pong mechanism (*12*):

$$\alpha\text{-Ketoglutarate} + \text{L-asparate} \rightleftharpoons \text{L-glutamate} + \text{oxalacetate}$$

When the enzyme in the adelhyde (PLP) form reacts with an excess of L-aspartate in the absence of α-ketoglutarate, oxalacetate is produced as many moles as the enzyme contains transaminationable active centers.

In this work we determined the effects of immobilization on the maximum velocity, biologically active PLP, and transaminationable active centers of AspAT.

Materials and Methods

Reagents

Bovine serum albumin, decanal, flavine mononucleotide (FMN), L-asparate, α-ketoglutarate, malate dehydrogenase (15 μkat/mg), NADH, and PLP were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Sepharose CL 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden, and cyanogen bromide was a product of Aldrich Chemcial Company, Milwaukee, USA. Oxalacetate was supplied by Fluka Ag., Buchs SG, Switzerland, and *N*-ethyl(2,3-^{14}C)-maleimide (10 mCi/nmol) by the Radiochemical Center, Amersham, England.

The bioluminescence reagent, consisting of a luciferase and an FMN : NADH oxidoreductase from *Beneckea harveyi*, was obtained from LKB-Wallac, Turku, Finland. The α-subform of cytosolic AspAT was purified from pig hearts according to Martinez-Carrion et al. (*13*).

Immobilization of the Enzyme

Sepharose CL 4B was activated with CNBr as described by Korpela and Kurkijärvi (*14*). The amount of CNBr was 0.2 g/2 g of moist gel. The washed activated agarose was suspended into 4 mL of 0.2*M* potassium phosphate, pH 7.0, containing 6 mg of the purified AspAT. The suspension was gently shaken at 4°C for 20 h. The AspAT-agarose conjugate formed was washed with 50 volumes of the coupling buffer, and then with 50 volumes of the same buffer containing 1*M* NaCl. The washed enzyme gel was stored at 2°C suspended in the latter solution.

Protein Determinations

The soluble protein was determined by the biuret assay with bovine serum albumin as the standard. The determination of bound protein was checked with *N*-ethyl(2,3-^{14}C)-maleimide labeled AspAT (*15*). It was shown that the modification by the maleimide did affect neither the activity nor the immobilization as compared to the unmodified AspAT. About 2% of the total protein (6 mg) in the coupling mixture was labeled, and the bound radioactivity was measured with an LKB-Wallac Ultrobeta scintillator.

Enzyme Assays

The reaction was followed in the direction from L-asparate and α-ketoglutarate to oxalacetate and L-glutamate to oxalacetate and L-glutamate at 25°C by photometrically measuring the ketoacid formed. The molar absorptivity of oxalacetate at 255 nm was $9.0 \times 10^2\ M^{-1}\text{cm}^1$. Three μg of soluble enzyme were used in 3 mL of 0.1*M* potassium phosphate buffer, pH 8,0, containing the substrates. The absorbance measurements were performed with a Gilford 2200 spectrophotometer equipped with Beckman DU monochromator. The activity of immobilized AspAT was measured with a stirred batch reactor (*16*). Forty μL of AspAT–agarose suspension (equalling 20 mg moist enzyme–gel) were pipeted into a reaction volume of 13 mL.

The maximum reaction rate was determined by varying the concentration of one substrate at five fixed concentrations of the other and by replotting the slopes of the primary Hanes plots as a function of the concentration of the fixed substrate. The ping-pong reaction shows a straight line, which has a slope of $1/V$. The K_m values can be calculated from the intersections in the ordinate of both the primary and secondary plots (*12*). For soluble enzyme, the concentration of L-aspartate varied from 0.5 to 20 m*M* at 0.045, 0.15, 0.45, 1.5, and 3.0 m*M* concentrations of α-ketoglutarate, or the concentration of α-ketoglutarate varied from 0.045 to 10 m*M* at 0.5, 1.0, 5.0, 10, and 20 m*M* concentrations of L-aspartate. The values for immobilized AspAT were 0.5–40 m*M* at 0.23, 0.46, 0.90, 2.3, 4.6 m*M*, and 0.46–10 m*M* at 2.3, 7.5, 14, 20, and 40 m*M*, respectively.

Enzyme-Bound Pyridoxal 5'-Phosphate

To ascertain that the enzymes were thoroughly in the aldehyde form they were first incubated in 1 m*M* α-ketoglutarate at room temperature. PLP was released by incubating the enzyme preparations in 5.5% (w/v) trichloroacetic acid at 50°C, and the quantities of the coenzyme were measured fluorometrically (Perkin-Elmer MPF-2A fluorometer) as cyanide complex (*17*).

Transaminationable Active Centers

From 10 to 40 mg of moist AspAT-agarose conjugate were first incubated at room temperature in 0.1*M* potassium phosphate, pH 8.0, containing 0.1 m*M* α-ketoglutarate. Thereafter, the enzyme–gel was washed slowly with 10 mL of 0.1*M* potassium phosphate, 1*M* NaCl, pH 8.0, followed with 10 mL of the same buffer without the salt. After washings the enzyme was incubated for 2 min in 0.5 mL of 0.1*M* potassium phosphate, pH 8.0, containing 10 μmol of L-aspartate, 1 nmol of NADH, and 50 nkat of malate dehydrogenase. The NADH consumption in the malate dehydrogenase reaction (= produced oxalacetate in the AspAT–reaction) was measured by using the bacterial bioluminescence reaction, which is well documented in the NADH monitoring at pmole levels (*18*). Finally, 10 μL of 2 m*M* FMN, 10 μL decanal (0.1 mg/mL of isopropanol), and 10 μL of the reconstituted bioluminescence reagent were added. The light emission was measured in LKB-Wallac 1250 luminometer and registered on a potentiometric 2210 Chart Recorder (LKP Produkter, Bromma, Sweden). The NADH and oxalacetate standards were measured similarly.

Results and Discussion

The kinetic measurements with immobilized AspAT showed linear Hanes plots intercepting on the ordinate, which is typical of the ping-pong reaction mechanism (Fig. 1). With lower substrate concentrations than indicated in Fig. 1, the plots were curved, which was not found with the soluble enzyme. Similar results were observed by varying the concentration of α-ketoglutarate at fixed L-aspartate concentrations. The deviations from linearity in enzyme kinetic plots at low substrate concentrations with immobilized enzymes have been attributed to mass transfer limitations, or to differences between the microenvironments of the bound enzyme molecules (*19*). The pH profile of AspAT did not change significantly upon immobilization.

The calculation of meaningful data from curved plots has been questioned (*20*). In this work, the use of rather high substrate concentrations produced linear plots (Fig. 1), making the determination of the slopes quite accurate, although there remains a possibility that the calculated K_m values are invalid below the used substrate concentrations. The secondary plot of the fixed substrate concentration, multiplied by the slopes in Fig. 1 on the ordinate versus the fixed substrate concentration, is shown in Fig. 2. The calculated K_m values and maximum velocities of soluble and immobilized AspAT are presented in Table 1. It has been shown that

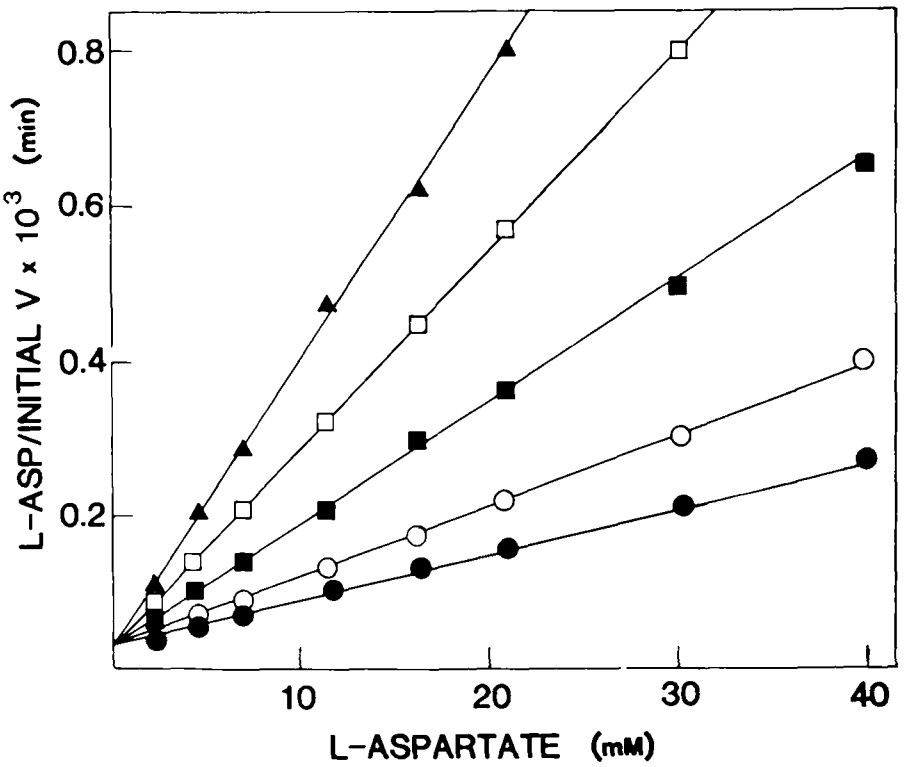

Fig. 1. The effect of L-asparate concentration on the activity of AspAT–agarose conjugate. Assays were performed at five fixed α-ketoglutarate concentrations. ●, 4.6 m*M*; ○, 2.3 m*M*; ■, 0.90 m*M*; □, 0.46 m*M*; ▲, 0.23 m*M* α-ketoglutarate. The lowest L-asparate concentrations are left out from the figure (see the text). The rotation speed of the stirrer was 630 rpm.

immobilization on collagen films does not significantly affect the intrinsic kinetic properties of AspAT, diffusion limitations alone accounting for the changed enzyme affinities toward its substrates (*10, 15*). Assuming the same situation, the present work also supports the postulation that with two-substrate enzymatic reactions the mass transfer effects are more significant with the substrate having the higher affinity (*21*). Although, the external diffusion limitations were tested to be negligible with the used rotation speed of the stirrer in the substrate concentrations presented in Fig. 1, the internal mass transfer could still be limiting.

Determination of Enzyme-Bound Pyridoxal- 5'-Phosphate

Cytosolic AspAT is a dimeric enzyme having a molecular weight of 92,688 and containing two PLP per enzyme molecule (22). The purified soluble enzyme preparation was found to contain 14.8 nmol of PLP/mg of protein (Table 1), which correlates to a purity of 70%.

Measurement of the coenzyme in the immobilized enzyme required to lengthen the incubation time in trichloroacetic acid from 15 min to 2 h for complete release of PLP. This longer incubation time is reasonable since bound proteins are com-

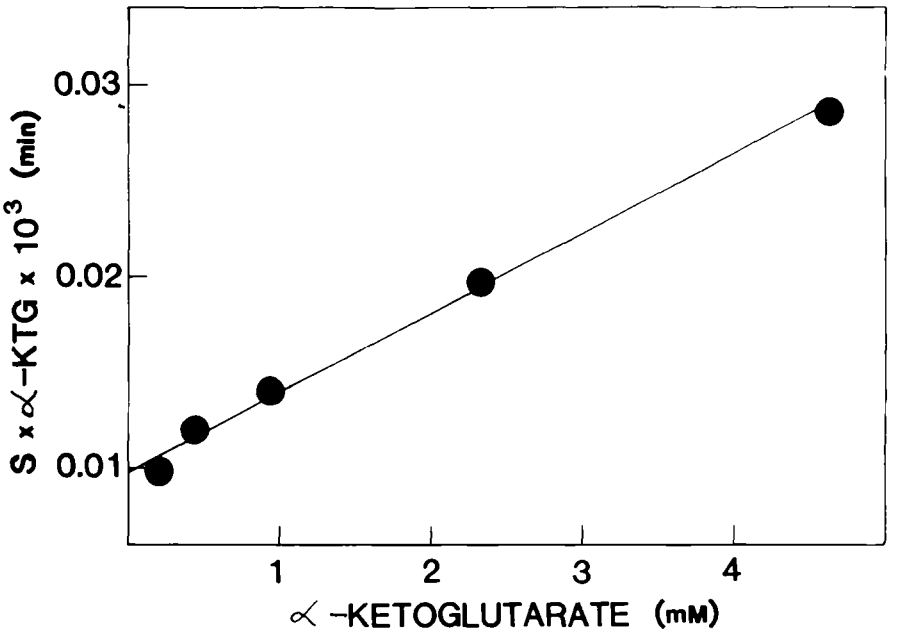

Fig. 2. Replot of the fixed substrate concentration multiplied by the slopes(s) on the ordinate versus the fixed substrate concentration for the data shown in Fig. 1.

TABLE I
Comparison of the Properties of Soluble and Immobilized Aspartate Aminotransferase

Protein, mg	6.0	5.4	90
Specific activity, μkat/mg	3.50(2.90)[a]	1.60 (0.85)[a]	46 (29)[a]
PLP, nmol/mg	14.8	12.3	83
Activity per PLP, ukat/nmol	0.24	0.13	54
Activity centers, nmol/mg	14.8	8.3	56
Turnover number, ukat/nmol	0.24	0.19	80
$K_m^{\alpha\text{-ktg}}$, m*M*	0.8 (0.6)[b]	2.4	—
$K_m^{\text{L-asp}}$, m*M*	4.0 (3.9)[b]	4.2	—

[a]The activities were measured with standard concentrations of the substrates (both 20 m*M*).
[b]From Nisselbaum, J. S., and Bodansky, O. (1966), *J. Biol. Chem.* **241**, 2661.

monly more stable against denaturants than the soluble ones. As shown inTable 1, the coenzyme content of immobilized protein was 17% lower than that of soluble one. However, the activity of immobilized AspAT did increase neither after overnight incubation in 1 m*M* PLP solution nor after the coenzyme was used as an additive in the coupling solution. It has been shown that immobilized AspAT, prepared from the apoprotein, exhibits (after reconstitution with PLP) equal activity to AspAT immobilized as holoenzyme. This indicates that the lysine residues (numbered 258) at the active sites of the enzyme are quite unreactive, for steric hindrances, to CNBr-activated agarose both in apo- and holoenzyme (*22*). In this

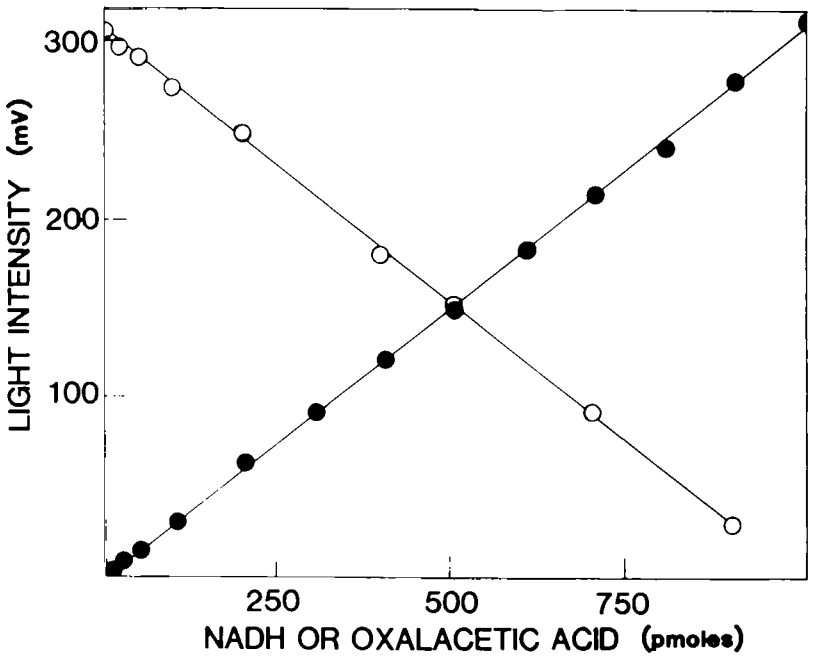

Fig. 3. Peak light intensities as a function of the amount of NADH (filled circles) and oxalacetate (open circles). See Materials and Methods for experimental system.

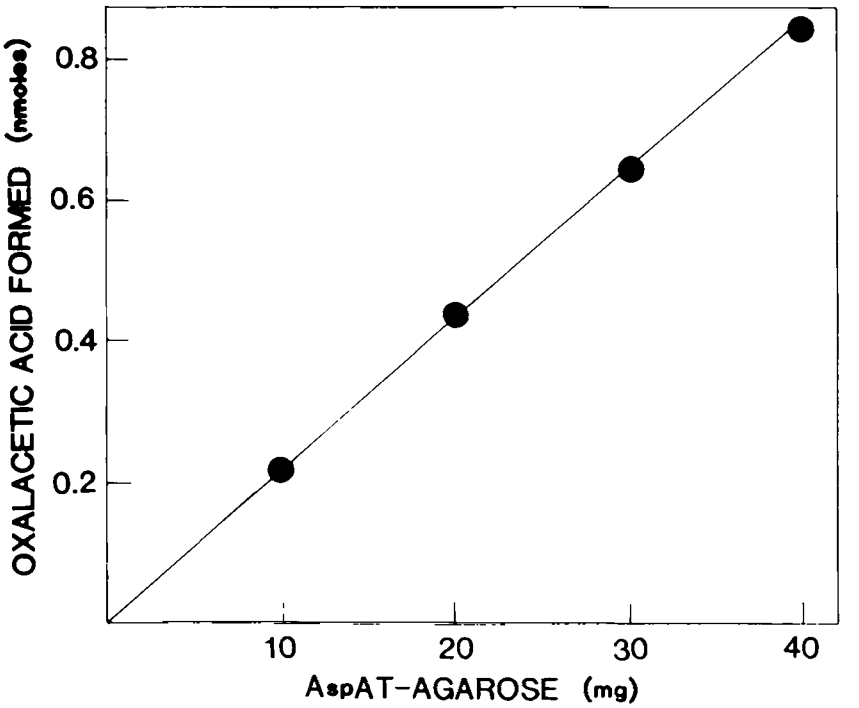

Fig. 4. Formed oxalacetate as a function of the amount of immobilized AspAT in the half-reaction of the enzyme. The reaction was performed in excess of L-aspartate (20 m*M*) and in absence of a α-ketoglutarate. Oxalacetate was determined from Fig. 3.

work, the most plausible explanation of the loss of PLP upon immobilization is that the coupling mixture contained a small quantity of CNBr, which as nucleophilic reagent had reacted with the lysine bound PLP. This conclusion is supported by our observation that the thoroughness of the washing after the activation of the gel increased the recovery of activity, but did not affect the bound protein in immobilization.

Determination of Active Centers

The bioluminescence assay responded linearly to NADH from 1 pmol to 1 nmol, and malate dehydrogenase converted quantitatively 10–900 pmol of oxalacetate to malate with 1 nmol of NADH in the used reaction conditions (Fig. 3). Standard deviations for repeated determinations (n = 10) were less than 3%.

Figure 4 presents the formed oxalacetate as a function of the AspAT–agarose conjugate in the excess of L-aspartate. The immobilized preparation contained 8.3 nmol of active centers/mg of protein (Table 1). Because the soluble AspAT was of α-subform, the quantity of active centers was assumed to be the same as the number of enzyme-bound PLP (*23*, Table 1). The transaminationable active centers of the soluble enzyme were also attempted to measure, but the results were inaccurate because the method demanded careful dialysis to remove α-ketoglutarate. This caused 10–20% inactivation of the aminotransferase.

The oxalacetate can also be measured both by the absorbance at 255 nm and by the absorbance at 340 nm after the malate dehydrogenase reaction (*22*), but these methods are at least 100–1000 times less sensitive than the presented bioluminescence system. Parvin et al. (*24*) have reported an assay for oxalacetate based on the citrate synthase-catalyzed conversion of radiolabeled acetyl-CoA to citrate, which is as sensitive as the bioluminescence method here, but is much more complicated to perform.

Since there were rather many biologically active PLP molecules, compared to the number of active centers in the immobilized AspAT (Table 1), it could be thought that the nonreactive enzyme bound coenzymes were capable of utilizing their active counterparts, so that they could replace the amine (PMP) forms of the coenzyme molecules in the active centers. This requires that the coenzyme molecules be easily removable from their binding sites. This model, however, is an unlikely one because repeated measurements with appropriate washings showed the same number of active centers (n = 10, sd less than 5%).

Comparison of the Coupling Recoveries

The coupling efficiency was 90%, which means that the gel preparation contained 2.7 mg of protein/g of agarose (Table 1). When measured with standard concentrations of the substrates (both 20 mM), the coupling recovery of specific activity was 29% (Table 1, in parentheses). Comparison of the maximum velocities, extrapolated from the appropriate experimental data (see Figs. 1 and 2), yielded a coupling recovery of 46% (Table 1). The measurements in the standard conditions showed 37% apparent decrease in the coupling recovery of specific activity as compared to the corresponding value calculated from the maximum velocities (46–29/46 × 100).

The activity of soluble AspAT with standard concentrations of the substrates was 83% of the extrapolated maximum rate (see Table 1), which is in good agreement with the theoretical value (81%) calculated from the rate equation of the two-substrate ping-pong reaction:

$$v/V\% = (ab/ab + bK_m^a + aK_m^b) \times 100$$

V indicates the maximum rate, v the initial rate, and a and b the concentrations of the substrates. Correspondingly, the activity of bound AspAT with the standard concentrations of the substrates was only 53% of the maximum rate (Table 1), while the calculation from the rate equation yielded a value of 75% (the K_m values for bound enzyme in Table 1 were used). At high concentrations, α-ketoglutarate is known to form a dead-end complex with AspAT (*25*). Although the soluble enzyme did not show any detectable substrate inhibition under the standard conditions, the conformation of AspAT could be changed upon immobilization favoring the formation of the dead-end complex.

As calculated on the basis of the enzyme-bound coenzyme, a very high coupling recovery (83%, Table 1) was obtained. This was apparently much above the actual value of the specific activity of immobilized AspAT. Therefore, any direct determination of coenzymes in the immobilized preparations, at least in the case of AspAT and other PLP enzymes, which usually have their coenzymes rather tightly bound to the apoprotein, poorly illustrates the quantity of bound active enzyme. If the binding of the coenzymes is sensitive to small conformational changes in the apoprotein, the release of coenzyme may indicate the amount of active enzyme in the support. If it is supposed that all PLP sites are reactive, in the immobilized preparation they should function with an average catalytic efficiency of only 54% (Table 1).

The direct measurement of the transaminationable active centers was totally free from any complications caused by mass transfer limitations. The coupling recovery obtained with this system was 56%, which was 10% units higher than that found in the comparision of the maximum velocities of soluble and bound AspAT (Table 1). On the other hand, however, it is possible that not all active centers of the immobilized enzyme had equal catalytic rates because of enzyme–support interactions or internal mass transfer limitations. For example, the active centers having turnover numbers several orders lower than these of the native enzyme can be measured by the direct method, while they are omitted from kinetic measurements. If the maximum catalytic rate is presumed to be obtained from the extrapolated maximum velocities, it is calculable that each transaminationable center had an average of 80% of the turnover efficiency of the soluble AspAT (Table 1).

The average turnover number of AspAT would also have been decreased upon immobilization when the general properties of the enzyme are taken into account. The cytosolic AspAT occurs at least in there subforms (α, β, γ), all of which contain two PLP sites per enzyme molecule. Only the α-subform is fully active, while the other forms are more or less degenerate, having only partial activity (*23*). Immobilization could cause transformation of the α-subform to the less active forms.

In conclusion, immobilization of AspAT on Sepharose showed rather high coupling recovery of activity. Neither enzyme–matrix interactions nor internal mass transfer limitations had significant effects on the turnover efficiency of bound

catalytically active enzyme. This makes agarose beads a very useful matrix for immobilization. We are currently studying how to decrease the degree of inactivation by protecting the enzyme with appropriate compounds during the immobilization procedure.

Acknowledgment

The authors wish to thank LKB-Wallac, Turku, Finland for kindly donating the luminometer and the bioluminescence reagents.

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