FAST CONFORMATIONAL CHANGES AT THE ACTIVE SITE OF ASPARTIC AMINOTRANSFERASE

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1. Introduction

It was observed that conformational changes occur during the catalytic activity of aspartic-aminotransferase (AAT),* and following the binding of pseudo substrates [1–3]. The general relevance of conformational changes in enzyme catalysis has been discussed in many reviews [4,5]: in some cases the study of the pH dependence of the relaxation times characteristic of such conformational transitions led to the identification of dissociable groups involved in the process: a classical example is the study of ribonuclease [6].

At least two dissociable groups are known to be present at the active site of AAT: one contributed by the vitamin B_6 chromophore [7,8], the other by the protein [9]. In the present paper we investigated conformational transitions possibly connected with protonation of the latter group. To this end conditions were chosen (0.1 M NaCl, no buffer) where the spectral change of the B_6 chromophore is relatively slow ($\tau \gtrsim 1$ msec) so that it does not interfere with faster relaxations the latter were monitored by the addition of a fast pH indicator (chlorophenol red or phenol red) which does not interact with the protein.

The observed relaxation effects can be interpreted assuming a fast conformational transition coupled with the a co-operative uptake of two protons by the

* *Abbreviation*: AAT = Aspartic aminotransferase.

protein. The pK of this protonation is 6.25 ± 0.05, i.e. it coincides with that observed in nuclear magnetic resonance experiments [9] on the binding site of anions and attributed to the essential hystidyl residue. A similar relaxation effect is visible also in the presence of the substrate L-aspartate, but it disappears in the presence of an excess of the specific inhibitor succinate.

2. Experimental

Cytoplasmic L-aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) from pig heart purchased from Whatman, Ltd., was desalted by dialysis against water followed by chromatography on a Sephadex G-10 column. The remaining ionic contaminants were removed by chromatography on a mixed-bed ion-exchange column according to Timasheff [10]. The pH of the deionized solution, containing approximately 15–20 mg/ml of protein, was between 5.7 and 5.9. The apparent pK of the active site chromophore of the thoroughly deionized enzyme was 5.4. (The apparent pK is defined as $pK_{app} = pH - \log_{10} (\alpha/(1-\alpha))$: where α is the molar fraction of the enzyme showing absorption maximum at $\lambda = 435$ nm). The concentration of the active site chromophore was calculated from the absorbancy at the isobestic point for the spectral change induced by protonation (390 nm) using a molar extinction coefficient of $2900 \ M^{-1} \ cm^{-1}$.

Phenol red (a commercial sample from B.D.H.) was purified by reprecipitation [11]. All other reagents were from Sigma Inc. or from Merck A.G. Equilibrium dialysis was performed with a Visking membrane at 4°C for 12 hr.

Temperature jump experiments were carried out in a 'Messanlagen T. J. Transient Spectrophotometer Type SBA7' using a mercury lamp and a 1 ml micro-cell, thermostated at $18.5 \pm 0.5°C$. The temperature jump was 4°C. The output of the spectrophotometer was memorized on a digital transient recorder (Bio-matic mod. 802). The registered punched tape was analyzed by computer with best fitting procedures.

Each experiment was repeated several times. The experimental errors reported under Results and discussion include both instrumental and repro-ducibility errors.

Results and discussion

Since, in the present work, the attention was fo-cused on the protonation at protein sites other than the coenzyme chromophore, conditions were first studied in which the protonation of the latter would not interfere. pH dependent relaxation effects involv-ing spectral changes of the coenzyme do occur and are strongly dependent upon the anions present in solution. A systematic study of these effects will be reported elsewhere. In the presence of 0.1 M NaCl and in the absence of buffer, the relaxation time observed at the chromophore wavelengths (366 or 435 nm) was always larger than 1—2 msec. Under these conditions and using chlorophenol red as indi-cator we observed a faster process: fig.1 shows a relaxation effect as plotted by the computer on ana-
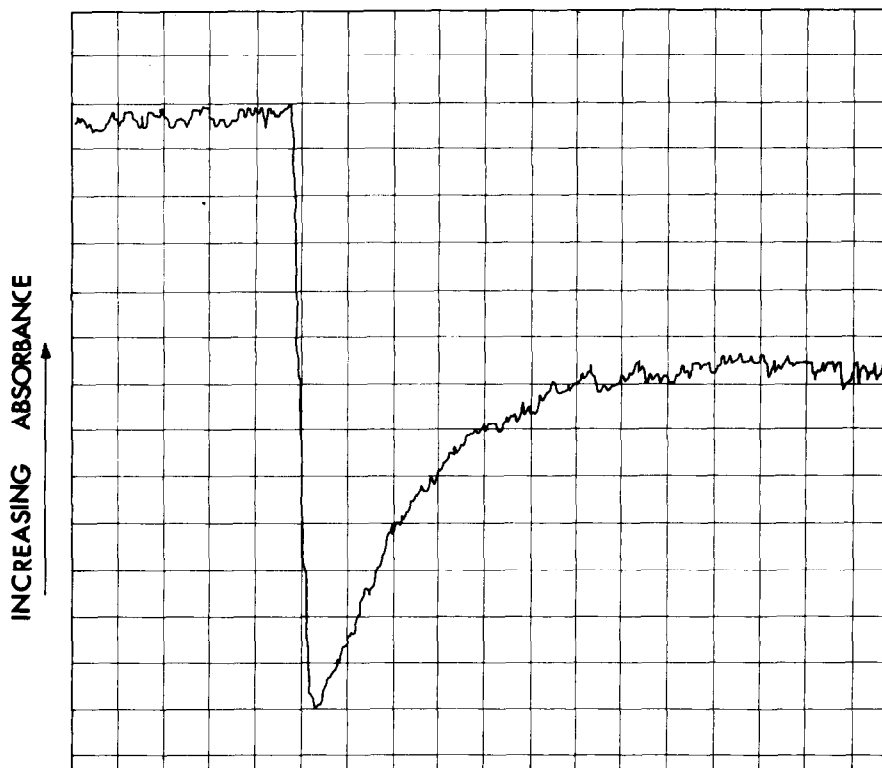

Fig.1. Relaxation effect coupled with proton uptake by the AAT protein, and monitored by a pH indicator (chlorophenol red). AAT was 9.5 mg/ml in NaCl 0.1 M pH 5.95 and chlorophenol red was 5×10^{-5} M; $\lambda = 578$ nm (absorption maximum of the non-protonated form of the indicator); 4 mV/div and 0.1 ms/div. 10 mV correspond to a change in optical density of 10^{-3}.

lysis of the experimental data punched on tape. Following the very rapid approach to equilibrium between the indicator and the dissociating groups of the protein, a relaxation effect is observed with a time constant of 250 μs, accompanied by a decrease of H^+ concentration.

This value is in the range of the relaxation times of some known conformational changes in proteins [4]. The observed relaxation effect is connected with an intramolecular event, presumably a conformational change, because other interpretations bared on inter-molecular interactions can be excluded for the following reasons:

(a) The observed effect cannot be attributed to the reversible binding of the indicator to the enzyme because (i) in equilibrium dialysis experiments between indicator (0.05 mM chlorophenol red) and 0.1 mM enzyme, the concentration of the indicator inside and outside of the dialysis tube was the same within the experimental error ($\sim$ 1%), suggesting that the indicator does not bind to the enzyme or that it binds with an affinity constant much too low to account for the large relaxation effects observed; (ii) in the temperature jump experiments the relaxation time was independent, within the experimental error, from the enzyme and indicator concentrations (from 3 to 16 mg/ml for the enzyme and from 0.01 to 0.1 mM for chlorophenol red); (iii) the catalytic activity of the enzyme assayed under standard conditions [12] was not affected by the presence of the indicator even at concentrations 10 times greater than those used in temperature jump experiments;

(b) Self association of the enzyme to give oligomers can be ruled out from the observed independence of relaxation times from the enzyme concentration within the range covered by our experiments. Moreover, the relaxation amplitude increased linearly within the same range:

(c) An interaction between the enzyme and NaCl cannot be directly involved in the effect because the relaxation time was found to be independent of NaCl concentration in the 0.02 to 0.5 M range; moreover the observed effect is much slower than expected for this type of interaction.

Similar arguments have already been used to ascribe relaxation effects observed with other enzymes to conformational changes [4].

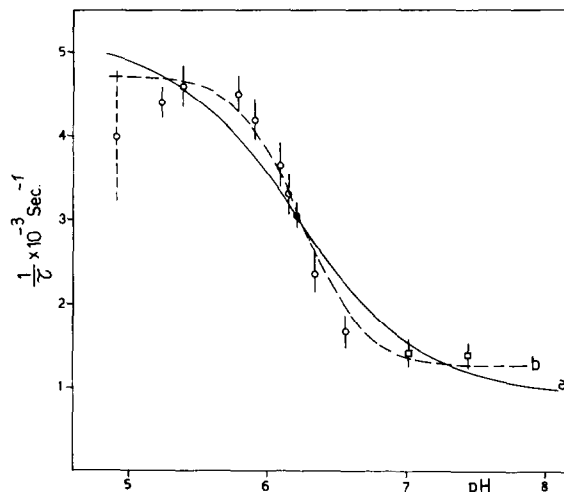The pH dependence of the relaxation times is

Fig. 2. pH dependence of the reciprocal relaxation times for AAT conformational change. The indicator was: (□) = phenol red, (○) = chlorophenol red. Other conditions, except pH, as in fig. 1. The lines are the best fit assuming that the isomeriza-tion is coupled with: (a) (continuous line) = uptake of one proton; (b) (dashed line) = highly co-operative uptake of 2 protons.

shown in fig. 2. The pH was varied by adding NaCl at different pH to a water solution of the enzyme which acted as buffer the final adjustment being made with dilute NaOH or HCl. The resulting ionic strengh was 0.1 M and the enzyme concentration generally 9.5 mg/ml. The indicator was chlorophenol red at pH $\leqslant 7$, phenol red at pH $\geqslant 7$. The curve in fig. 2 corresponds to a steep titration curve with $pK = 6.25 \pm 0.05$.

The relaxation amplitudes show a maximum at pH$\sim$6.

The following findings indicate that the postulated conformational change involves the active site of AAT.

(1) Under conditions identical to those of fig. 1, no relaxation effect was observed with the ψ form of *AAT* [13], in which the coenzyme is bound in an 'inactive' mode.

(2) The addition of the specific inhibitor Na succinate (10 mM) eliminated the effect; the effect vanished also in the presence of 0.5 M NaCl. This indicates that the specific binding of anions at the active site [8] hinders the conformational change.

(3) Experiments were performed on 9×10^{-5} M AAT in the presence of 70 mM L-aspartate. pH changes were monitored with chlorophenol red. It is

known [2] that under these conditions the enzyme is almost completely associated with the substrate and actively turning over. An effect quite similar to that reported in fig.1 was observed. The relaxation time was $300 \pm 30 \, \mu s$, and was independent of pH between 5.5 and 7.0. A more detailed study of this effect and its connection with catalytic steps is under way.

An attempt to fit the data of fig.1 with a simple model of conformational isomerization coupled to the proton dissociation of one group [6] gave a curve (curve (a) in fig.2) which is *not* in agreement with the observed data. Curve (b) was obtained assuming that the isomerization of two groups is coupled with the dissociation according to the following scheme:

$$EHH_1 \rightleftharpoons EHH_2 \rightleftharpoons EH + H^+ \rightleftharpoons E + 2H^+$$
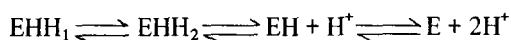
Assuming that the dissociation of the two groups is highly co-operative and faster than the isomerization, the scheme reduces to;

$$EHH_1 \underset{k^-}{\overset{k^+}{\rightleftharpoons}} EHH_2 \underset{K_D}{\rightleftharpoons} E + 2H^+$$
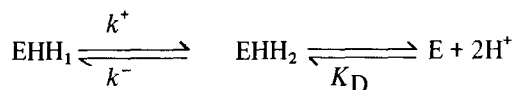
where k^+ and k^- are the kinetic constants for the isomerization step and K_D is the equilibrium dissociation constant for the co-operative two-fold protonation. The relaxation time is then

$$1/\tau = k^+ + k^- (1 - K_D/[H]^2)^{-1}$$

This expression fits the data with $pK_D = 2 \times$ (6.25 ± 0.5) and $k^+ = 1.30 \times 10^3 \, sec^{-1}$, $k^- = 3.42 \times 10^3 \, sec^{-1}$.

The data are also compatible with a less co-operative dissociation of more than two groups.

Finally the nuclear magnetic resonance data [9] for anion binding to AAT at low anion concentration have a pH dependence which is quite similar to that shown in fig.2; the pK is the same and the experimental data essentially overlap; the precision of the NMR experiment, however, does not allow to distinguish between a single and a cooperative protonation.

An essential hystidyl residue at the active site of AAT was shown to be responsible for the protonation observed in the nuclear magnetic resonance experiments. It seems probable that the dissociation of this hystidyl group is coupled to the conformational change reported here. The cooperativity in the proton uptake suggested by the present data could be explained if the hystidyl residue were linked (e.g. by hydrogen bond) to neighbouring groups forming a proton relay system. We cannot exclude, however, co-operative interactions with some distant dissociating groups.

Acknowledgements

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