Thermal Inactivation Studies of Normal and Variant Human Erythrocyte Carbonic Anhydrases by using a Sulphonamide-Binding Assay

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Heat-inactivation studies were carried out on the two primary erythrocyte carbonic anhydrase isoenzymes, CA I and CA II, and the secondary isoenzyme of CA I, CA I(+1). In addition, two genetic variants of human isoenzyme CA I, CA Id Michigan (100 Thr→Lys) and CA If London (102 Glu→Lys), and one variant of isoenzyme CA II, CA IIh (251 Asn→Asp), were similarly analysed. The first-order rate constants and Arrhenius plots for these six enzyme forms showed that (1) isoenzyme CA II is more heat-stable than CA I, (2) isoenzyme CA I(+1) is less heat-stable than CA I, (3) the variants CA IIh and CA If London are less heat-stable than the normal enzymes, and (4) isoenzyme CA Id Michigan is more heat-stable than normal CA I. From the values of the slopes of the Arrhenius plots, the energy of activation (E_a) for each isoenzyme and isoenzyme variant was determined, and the following thermodynamic activation parameters were calculated at 55°C: the free energy of activation (ΔG^*), the activation enthalpy (ΔH^*) and the activation entropy (ΔS^*). The ΔG^* for the enzymes shows a relative constancy with compensating variation in ΔH^* and ΔS^* . When the values for ΔH^* are plotted against ΔS^* , an increase in ΔH^* involves a concomitant increase in ΔS^* .

Two primary isoenzymes of the zinc metalloenzyme, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) are normally present in human erythrocytes (for recent reviews see Lindskog *et al.*, 1971; Carter, M. J., 1972). These isoenzymes, designated CA I (or CA B) and CA II (or CA C), are the products of two autosomal loci which are probably linked (Carter, N. D., 1972; DeSimone *et al.*, 1973). On the basis of their markedly different specific CO₂ hydratase activities, isoenzymes CA I and CA II have also been termed the low-activity enzyme and the high-activity enzyme respectively. The complete primary sequences of both human isoenzymes have now been completed (Andersson *et al.*, 1972; Laurent-Tabusse *et al.*, 1972; Lin & Deutsch, 1973; Henderson *et al.*, 1973), and the three-dimensional structures of both enzymes have been determined at the 0.2 nm (2.0 Å) level (Liljas *et al.*, 1972; K. Kannan, personal communication).

A number of secondary isoenzymes, presumably derived from the primary molecules, have been described from human haemolysates (cf. Funakoshi & Deutsch, 1968; Tashian, 1969), the most prominent of which is a secondary isoenzyme of CA I, designated by us as CA I(+1) and by others as CA A or CA b₁. There is evidence that isoenzyme CA I(+1) is either a conformational isoenzyme (conformer) of CA I (Headings & Tashian, 1971), or results from the deamidation of amide residues (Funakoshi & Deutsch, 1969), or is formed by a combination of these processes (Bouthier *et al.*, 1973).

To date, some nine rare electrophoretic variants of human isoenzyme CA I have been described (cf. Tashian, 1969; Carter *et al.*, 1972, 1973; Moore *et al.*, 1973; R. E. Tashian, unpublished work), and one polymorphic variant of isoenzyme CA II has been reported (Moore *et al.*, 1971).

This report is part of a study on the stabilities to heat and proteolysis of normal, variant and secondary forms of various homologous carbonic anhydrases, with respect to known structural differences. This first report concerns the process of thermal inactivation in normal human isoenzyme CA I, its secondary isoenzyme, CA I(+1), the CA I variants, CA Id Michigan and CA If London, and CA II, and the CA II variant CA IIh [designated CA H by the original authors (Moore *et al.*, 1971)]. The electrophoretic patterns of these carbonic anhydrases are shown in Plate 1. Although the structural modifications responsible for the formation of isoenzyme CA I(+1) are not known, the exact amino acid substitutions have been determined for CA Id Michigan (Tanis *et al.*, 1973), CA If London (Carter *et al.*, 1973) and CA IIh (Lin & Deutsch, 1972).

To monitor the thermal inactivation of carbonic anhydrase, a reliable quantitative assay was required. An assay which is used as a routine to measure carbonic anhydrase activity involves the hydrolysis of *p*-nitrophenyl acetate (cf. Whitney *et al.*, 1967; Armstrong *et al.*, 1966); however, this method did not meet our requirements as it is relatively time-consuming and not very reproducible, therefore an

alternative procedure was developed which utilized the property of sulphonamide binding to carbonic anhydrase.

It has been extensively reported that aromatic sulphonamides are specific, non-competitive inhibitors of carbonic anhydrase, exhibiting a high affinity for the enzyme (cf. Carter, M. J., 1972; Taylor *et al.*, 1970a). The kinetics and mechanisms of binding have been investigated by Taylor *et al.* (1970a,b). Of the many sulphonamides investigated, the azosulphonamides were of interest to this study, as they exhibit appreciable absorption in the visible spectrum (Coleman, 1968). In particular, the azosulphonamide, 7-acetamido-2-(4'-sulphamylphenylazo)-1-hydroxynaphthalene-3,6-disulphonate (Neoprontosil) is commercially available and has an absorption peak at about 500 nm which decreases in intensity on binding to carbonic anhydrase. It was found that this complex followed Beer's Law, making it possible to utilize this change in absorbance to measure carbonic anhydrase quantitatively. The method that was adopted was to measure the difference in absorption between bound and unbound inhibitor, a procedure which was reproducible, rapid and required relatively small amounts of enzyme. The validity of the technique was confirmed by comparison with an esterase (*p*-nitrophenyl acetate) assay.

Materials and Methods

Reagents

Neoprontosil was obtained from Winthrop Laboratories, New York, N.Y., U.S.A. All chemicals were reagent grade and supplied by Sigma, St. Louis, Mo., U.S.A.

Enzymes

Human isoenzymes CA I, CA I (+1), CA Id Michigan, CA II and CA IIh were prepared by the methods described by Tanis & Tashian (1971). CA If London was a gift from Dr. N. D. Carter, London Hospital Medical College, Turner St., London E.1, U.K. The concentration of purified enzyme was determined by measuring E_{280} and using molar absorbance values of 4.8×10^4 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ for the low-activity enzymes and 5.7×10^4 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ for the high-activity enzymes.

Enzyme activity

This was determined by measuring esterase activity towards *p*-nitrophenyl acetate in Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)-ethanesulphonic acid] buffer at pH 6.7. At this pH value there is no hydrolysis of ester in the absence of enzyme. The increase in E_{348} was monitored by using a

Beckman Acta II spectrophotometer equipped with an external recorder, by using an expansion facility such that 0.1 absorbance unit gave a full-scale response. The reaction was maintained at 20°C by means of a thermostatically controlled cooling unit. Stock solutions of 3 mM-*p*-nitrophenyl acetate in 3% (v/v) acetone and 50 mM-Hepes buffer, pH 6.7, were used. The procedure was to mix 0.5 ml of buffer and 0.3 ml of substrate in a 1 ml cuvette followed by equilibration at 20°C. The reaction was started by the addition of 10–30 μ l of a 10–15 μ M enzyme solution. Over this range, a linear relationship was obtained between enzyme concentration and absorbance increase with unit time.

Difference spectra

The difference spectra of the carbonic anhydrase-Neoprontosil complexes were determined with a Beckman Acta II recording spectrophotometer equipped with a flat-bed recorder. The cell block was maintained at a temperature of 25.0°C by a thermostatically controlled water bath fitted with a circulating pump. The spectra were recorded over the range 600–340 nm, and the wavelength and the absorption of the peak maxima were recorded. To ensure that the enzyme was present in the complexed form, an excess of Neoprontosil was used. A zero difference spectrum was obtained between reference and sample cuvettes containing Neoprontosil in 0.1 M-Hepes buffer at pH 7.5. The difference spectrum produced by inhibitor binding was obtained by adding a portion of enzyme to the sample cuvette and an equal volume of water to the reference cuvette so that the final concentration of enzyme was about 20 μ M and that of inhibitor 40 μ M.

The applicability of Beer's Law was confirmed at the wavelength of maximum absorbance difference of isoenzyme CA I, by using a Zeiss PMQ II spectrophotometer. The differential absorbance of enzyme-inhibitor complex was measured over the concentration range 1–30 μ M.

Thermal inactivation

The enzyme was incubated in a glass-jacketed vessel which was maintained at the required temperature to $\pm 0.05^\circ\text{C}$. Enzyme solution, 0.7 ml (approx. 15 μ M), was incubated in 0.01 M-Hepes buffer (pH 8.4 at 23°C). At timed intervals 100 μ l samples of enzyme were removed and transferred to a polythene micro-tube previously cooled in ice. To this container was added 20 μ l of a 120 μ M-sulphonamide solution in 0.05 M-Hepes buffer, pH 7.5. The tube was centrifuged at 15000 rev./min for 20 min to remove any colloidal material that was formed, and about 100 μ l of enzyme-inhibitor complex was transferred to a

EXPLANATION OF PLATE I

Starch-gel-electrophoresis patterns of normal and variant forms of human erythrocyte carbonic anhydrases I and II

Vertical electrophoresis was at 4°C in 20mM-sodium borate buffer, pH 8.52, and 0.3M-sodium borate bridge buffer, pH 8.0 (containing 30mM-NaCl), for 21 h at 8 V/cm. Gels were stained with 0.4% Nigrosin in methanol-acetic acid-water (5:1:5, by vol.). (a) Normal CA I and CA II pattern; (b) CA IIh (heterozygote); (c) CA Id Michigan (heterozygote); (d) CA If London (heterozygote). Isoenzymes CA I (+1) and CA If (+1) are secondary isoenzymes of CA I and CA If respectively (see the text).

micro-cuvette of 90 μ l capacity (Pyrocell Manufacturing Co., Westwood, N.J., U.S.A.), and after reaching ambient temperature the absorbance was measured against a reference solution of 100 μ l of 0.01 M-Hepes buffer, pH 8.4, plus 20 μ l of 120 μ M-sulphonamide. These measurements were made with a Zeiss PMQ II spectrophotomer by using micro-cell accessories.

Results

The azosulphonamide difference spectra of isoenzymes CA I and CA II between 340 nm and 600 nm are shown in Fig. 1, the negative sign indica-

ting a decrease in absorbance relative to free inhibitor. The enzyme concentrations were 22 and 20 μ M for isoenzymes CA I and CA II respectively, with a 100% molar excess of azosulphonamide, at pH 7.5. The six enzymes tested all gave a maximum negative change in absorbance between 485 and 490 nm, with peaks of positive sign at 550 and 350 nm. The change in molar extinction coefficient ($\Delta\epsilon$) for the complexes at 490 nm is listed in Table 1. The experiments to verify Beer's Law by using normal isoenzyme CA I gave a linear relationship between differential absorbance and concentration. The absorbances were measured at 490 nm over the concentration range 1-30 μ M.

Heat inactivation

Preliminary experiments indicated that the rate of inactivation varied with buffer type, molarity and pH. Precipitation of enzyme and non-linearity of inactivation rate was given with pH values at, or adjacent to, the pI values of the enzymes. Also more reproducible results were obtained by monitoring the range of 0-50% inactivation. To negate any effect of competitive binding by anion buffers as reported by Magid (1968), the cationic buffer Hepes was used. This buffer has the advantage of a much lower temperature-pH coefficient in comparison with Tris buffer. To facilitate a comparison of the various enzymes, a test pH of 8.4 at 23°C was chosen. Over the range of temperatures used for the heat-inactivation experiments, the actual pH range was 8.01-8.03. At these pH values, precipitation of enzyme was avoided, and all of the enzymes had an overall negative charge. For the two common forms of the enzyme, i.e. isoenzymes CA I and CA II, triplicate experiments were performed at the various temperatures used, and acceptable reproducibility was obtained. Because of shortage of material, experiments with the enzyme variants were in duplicate. To test the applicability of the inhibitor assay method, duplicate experiments with isoenzyme CA I at 57°C were performed by using this assay method and the esterase assay. The results for the

Fig. 1. Difference spectra at pH 7.5 of the Neoprontosil complexes of normal isoenzymes CA I (----) and CA II (—)

For details see the text.

Table 1. Change in molar absorbance of carbonic anhydrase isoenzyme complexes with Neoprontosil at 490 nm

For details see the text. In all cases λ_{max} . was at 488 nm.

Enzyme	$10^{-3} \times \Delta\epsilon$ ($l \cdot mol^{-1} \cdot cm^{-1}$)	Amino acid substitution of variant enzyme	Reference
CA I (normal)	7.7		
CA If London	7.7	102 Glu $\rightarrow$ Lys	Carter <i>et al.</i> (1973)
Ca Id Michigan	7.5	100 Thr $\rightarrow$ Lys	Tanis <i>et al.</i> (1973)
CA I (+1)	7.5		
CA II (normal)	10.3		
CA IIh	10.3	250 Asn $\rightarrow$ Asp	Lin & Deutsch (1972)

rate constants were $2.6 \times 10^4 \text{ s}^{-1}$ and $2.5 \times 10^{-4} \text{ s}^{-1}$ for the esterase assay and inhibitor assay respectively. Within experimental error these results are the same, and thus, although the inhibitor assay is a measure of the integrity of the binding site and not of catalytic activity as such, it can be used as a reliable index of activity. Because of differences in heat stability, isoenzyme CA I (+1) was tested over the range 51–54°C, whereas the other enzymes were measured over the range 55–58°C. The results of experiments at 55°C for the CA I, CA If and CA Id enzymes are shown in Fig. 2, and those at 56°C for the CA II and CA IIh enzymes are shown in Fig. 3. The slope values were obtained with a Compucorp 140 calculator by using a standardized linear-regression

Fig. 2. Heat degradation of normal isoenzyme CA I (○), isoenzymes CA If London (Δ) and CA Id Michigan (□), at 55°C in 10mM-Hepes buffer at pH8.0

For details see the text.

Fig. 3. Heat degradation of normal isoenzyme CA II (Δ) and isoenzyme CA IIh (■) at 56°C in 10mM-Hepes buffer at pH8.0

For details see the text.

program. The replicate values for the rate constants were averaged for use in the Figures and in the slope calculation. The results for all the experiments, in the form of Arrhenius plots, are shown in Figs. 4 and 5 for the low- and high-activity enzymes respectively. From the value of the slope, the Arrhenius activation energy (E_a) for each enzyme was calculated. The expression:

$$2.303 \log k = \frac{\Delta G^*}{RT} + 2.303 \log \frac{k_B T}{h}$$

derived from the theory of absolute reaction rates was used to calculate ΔG^* , the free energy of activation at $T = 55^\circ\text{C}$, where k (s^{-1}) is the rate constant for inactivation at this temperature, k_B is the Boltzman constant, h is Planck's constant, and R is the gas

Fig. 4. Arrhenius plots of the rate constants (k) for the heat degradation of normal isoenzyme CA I (○), isoenzymes CA If London (Δ), CA Id Michigan (□) and CA I (+1) (●)

For details see the text.

Fig. 5. Arrhenius plots of the rate constants (k) for the heat degradation of normal isoenzymes CA II (Δ) and isoenzyme CA IIh (■)

For details see the text.

Table 2. Thermodynamic activation parameters for carbonic anhydrase isoenzymes calculated at 55°C

For details see the text.

Isoenzyme or variant	$10^5 \times k$ (s ⁻¹)	E_a (kJ·mol ⁻¹)	ΔH^* (kJ·mol ⁻¹)	ΔG^* (kJ·mol ⁻¹)	ΔS^* (kJ·degree ⁻¹ · mol ⁻¹)
CA I (normal)	7.83	545.8	543.1	106.5	1.33
CA I (+1)	39.0	441.1	438.3	102.0	1.03
CA Id Michigan	4.67	624.5	621.8	107.9	1.57
CA If London	12.0	494.0	491.2	105.2	1.18
CA II (normal)	1.67	918.0	915.3	110.6	2.45
CA IIh	4.83	827.2	824.5	108.0	2.18

Fig. 6. Compensation plots of enthalpy (ΔH^*) and entropy (ΔS^*) of activation for the heat degradation at 55°C of normal isoenzyme CA I (○), isoenzymes CA If London (Δ), CA Id Michigan (□), CA I (+1) (●), normal isoenzyme CA II (▲) and isoenzyme CA IIh (■)

For details see the text.

constant. The rate constant at 55°C for isoenzyme CA I (+1) was obtained by extrapolation. The enthalpy of activation (ΔH^*) at 55°C was obtained from the Arrhenius energy (E_a) by using the following expression:

$$\Delta H^* = E_a - RT$$

The entropy of activation (ΔS^*) at 55°C was calculated from the expression:

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T}$$

These calculated thermodynamic parameters are shown in Table 2, and a compensation plot of enthalpy versus entropy is shown in Fig. 6.

Discussion

The Neoprontosil difference spectra of all the low-activity enzymes (the CA I forms) followed essentially the same pattern, having maximum

absorbance differences at about 490 nm, with a peak of opposite sign at 550 nm, of about 60–70% intensity. Both of the high-activity enzymes (the CA II forms) also gave difference spectra of the same type, with maximum absorbance differences at about 490 nm, and peaks of opposite sign at 550 and 350 nm. A comparison of the high- and low-activity enzymes shows common peaks at 550, 488, 410 and 348 nm differing only in value, except for the major peak at 488 nm, which also differed in shape. The most marked difference is shown in the shift of a minor peak, at 382 and 392 nm for isoenzymes CA I and CA II respectively. Coleman (1968) reported an increase in absorbance for the major peak at 500 nm for the human isoenzyme CA II–Neoprontosil complex. This is contrary to the results reported here, and also to the other mammalian enzymes studied by Coleman (1968). The change in molar extinction, $\Delta\epsilon$, at 490 nm shows a significant difference between the low- and high-activity isoenzymes, with mean values of 7.6×10^3 litre·mol⁻¹·cm⁻¹ and 10.3 litre·mol⁻¹·cm⁻¹ respectively. Although the substitutions of isoenzymes CA If (102 Glu→Lys) and CA Id (102 Thr→Lys) are relatively close to the active site, the absorbance differences within the low-activity enzymes are probably not significant. The addition of dithiothreitol to both high- and low-activity enzyme–Neoprontosil complexes increased by 30–50% the absorbance at 490 nm of these complexes, resulting in a decrease in differential absorbance (ΔE). Dithiothreitol had no effect on the absorption spectrum of free Neoprontosil, or on bovine carbonic anhydrase–Neoprontosil complex. As bovine carbonic anhydrase lacks a surface thiol group (Lindskog *et al.*, 1971), it was concluded that this decrease in ΔE was caused by the free thiol group of the human enzymes. It has been reported by Bergstén *et al.* (1972) and Erlich *et al.* (1973) that this thiol group is adjacent to the active-site cleft. A reaction with dithiothreitol would indicate that the thiol group was blocked, and this view was supported by the fact that the human enzymes did not react with 5,5'-dithiobis-(2-nitrobenzoic acid). A study of the active site topography of normal human isoenzyme CA II

indicates that the thiol group is sterically hindered, and this may be a contributing factor.

The results from the heat-inactivation experiments showed that under the test conditions the high-activity enzymes were significantly more stable than the low-activity enzymes. This is the converse of the results obtained by Edsall *et al.* (1966) and Carlsson *et al.* (1973) from denaturation studies with urea and guanidine hydrochloride. Also Riddiford *et al.* (1965) have shown that isoenzyme CA II was more unstable than isoenzyme CA I at pH values greater than pH 11. The lack of agreement between these studies and our results can probably best be explained by the different denaturation methods used. In the present study, denaturation was caused by heat at or near neutral pH in low-ionic-strength solutions, so that heat was the effective denaturant. The work of Edsall *et al.* (1966), Carlsson *et al.* (1973) and Riddiford *et al.* (1965) involved near-ambient temperatures, with high-ionic-strength or high-pH solutions as the denaturants. These latter denaturants have their initial effect on the enzyme-solvent interface, whereas the primary effect of heat is on the internal energy of the molecule. Therefore a different mechanism is involved in producing these denaturations, which could explain the observed differences in heat-stabilities. Also, it is noteworthy that Lindskog & Nilsson (1973) have shown that acid denaturation of human and bovine carbonic anhydrase results in incomplete exposure of tryptophan side chains, and that addition of urea or guanidine hydrochloride gives further spectral changes indicating greater unfolding of the molecules. Another contributing factor may be the different parameters used in monitoring denaturation; that is, activity versus change in u.v. absorption. It is possible that an enzyme can show changes in u.v. absorption and still exhibit activity. Carlsson *et al.* (1973) studied the denaturation of the human carbonic anhydrases by urea and guanidine hydrochloride by measurements of enzyme activity, optical-rotary dispersion and change in absorbance at 292 nm. They found that the absorbance change at 292 nm gave a higher initial rate than the other methods. These workers showed that for some denaturation conditions the overall reaction could not be described by first-order kinetics, although the reaction phase involving the initial loss of activity did follow first-order kinetics. It is also pertinent to inquire as to which of these denaturing methods relates to the stability of these enzymes *in vivo*.

The first-order rate constants and Arrhenius plots show differences within the low- and high-activity enzymes. The variant CA IIh is less heat-stable than the normal isoenzyme CA II. The amino acid substitution in isoenzyme CA IIh (i.e. 251 Asn→Asp) is nine residues from the C-terminus of the molecule (Lin & Deutsch, 1972) in a region far removed from the active site. The lower heat-stability of this variant

enzyme follows the trend shown by many reported enzyme and protein variants (cf. Motulsky & Yoshida, 1969; Huisman, 1969). However, the results from the one low-activity enzyme variant, CA Id Michigan, shows an exception to this trend, in that it is more heat-stable than the normal isoenzyme CA I. This result is in contrast with the CA If variant, which is less heat-stable than the normal enzyme. These findings are interesting because the amino acid substitutions in the variant enzymes are in close proximity to one another, being 100 Thr→Lys and 102 Glu→Lys for isoenzymes CA Id and CA If respectively. Although in both cases lysine is the substituted amino acid, isoenzyme CA If involves a charge change of negative to positive, whereas the change in isoenzyme CA Id is from nil to positive charge. The heat-stability of isoenzyme CA I (+1) indicates that it is markedly less stable than the normal and variant forms of isoenzyme CA I that were tested, and as an equilibrium could exist between isoenzymes CA I and CA I (+1) (Headings & Tashian, 1971) this may represent a possible pathway in degradation, assuming that the observed equilibrium actually occurs *in vivo*.

The findings that human isoenzyme CA II is more heat-stable than CA I, and that bovine isoenzyme CA II (also termed CA B) is more thermostable than both human enzymes (W. R. A. Osborne, unpublished work), suggest that perhaps all of the evolutionary homologous forms of CA II are more stable than the CA I isoenzymes. Thus in addition to the greater specific CO₂ hydratase activities of the CA II isoenzymes, and to the fact that these enzymes appear to be evolutionarily more conservative than the CA I forms on the basis of comparative sequence studies (Tashian *et al.*, 1972), it now also appears that the high-activity enzymes are characteristically more heat-stable than the low-activity forms.

The calculated free energy of activation (ΔG^*) for the enzymes shows a relative constancy, with compensating variation in activation enthalpy (ΔH^*) and activation entropy (ΔS^*). This exact compensation is shown in the plot of ΔH^* versus ΔS^* . It has been argued by Banks *et al.* (1972) that this compensation is an artifact of heat-denaturation experiments and the subsequent use of the transition-state theory to compute activation parameters. However, Exner (1964) has discussed the conditions necessary for the existence of a valid relationship between activation parameters, and following this Kemeny & Rosenberg (1973) have suggested that a range of variation in ΔH^* greater than 200 kJ/mol can establish the validity of a compensation law. In the data presented here, the variation in ΔH^* is about 480 kJ/mol, so it is reasonable to assume a valid compensation effect. Fig. 6 shows that an increase in ΔH^* involves a concomitant increase in ΔS^* . Therefore a statistical-mechanical interpretation of

the compensation plot would indicate that the more heat-stable enzymes have an increasing number of states associated with the activation process. The reasons for this are not readily interpreted in terms of single amino acid substitutions, or the more complex differences between the structures of isoenzymes CA I and CA II. An attempt to relate the structural changes in the mutant enzymes to the observed differences in their thermostabilities by using the analysis of nearest-neighbour amino acids (cf. Kabat & Wu, 1973) was inconclusive, perhaps indicating the empirical nature of this approach.

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