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Effects of Protein-Modifying Reagents on an Isoenzyme of Potato Apyrase

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Treatment of an isoenzyme of potato apyrase of high adenosine triphosphatase/adenosine diphosphatase (ATPase/ADPase) ratio with iodine, *N*-acetylimidazole or tetranitromethane inactivates the ATPase activity of this enzyme faster than its ADPase activity. There was protection by substrates with the two last-named substances. This and the appearance of nitrotyrosine suggests the participation of tyrosyl residues in both enzymic activities of potato apyrase. The participation of thiol groups is excluded by the insensitivity of apyrase to *p*-chloromercuribenzoate. Also, 2-hydroxy-5-nitrobenzyl bromide or carboxymethylation produce the same rate of inactivation of ATPase and ADPase activities. Substrates protect both activities from inactivation. Hydrogen peroxide and photo-oxidation inactivate ATPase activity faster than ADPase activity. There is no protection by substrates. Analysis of pH effects on $V_{max.}$ and K_m suggest different pK values for the amino acid residues at the ATP and ADP sites.

A diphosphohydrolase (EC 3.6.1.5), commonly called apyrase, has been described in several plant tissues (Kalckar, 1944; Mazelis, 1959; Nishikawa, 1966; Sikka & Das, 1964). The enzyme from potato tuber is the most studied apyrase. It splits ATP to AMP with sequential liberation of 2mol of orthophosphate. As a consequence, ADP is also a substrate (Traverso-Cori & Cori, 1962). It may also split other organic di- and tri-phosphates (Cori *et al.*, 1965; Miller & Westheimer, 1966). The rate of splitting of the standard substrates ATP and ADP may be very similar, or it may differ by a factor of 12 (Traverso-Cori *et al.*, 1970). Analysis of apyrase from different varieties of potatoes obtained by clonal reproduction (Traverso-Cori *et al.*, 1970) showed that in most cases the ratio between the rate of splitting of ATP and the rate of splitting of ADP (henceforth called the ATPase*/ADPase ratio) was fairly constant for enzyme preparations from one given variety throughout several purification steps. Since the final enzyme preparation was homogeneous in the ultracentrifuge it was proposed (Traverso-Cori *et al.*, 1970) that a single protein had both enzymic activities (i.e. ATPase and ADPase).

Electrophoretic studies also suggested that several isoenzymes of apyrase could exist, differing in their electric charge and in their ATPase/ADPase ratio (Traverso-Cori *et al.*, 1970).

Treatment of an apyrase of high ATPase/ADPase ratio with iodine at pH 8.0 produced a similar decrease of both activities (Traverso-Cori *et al.*, 1970). We have also observed that browning of our preparations by the action of tyrosinase led to a loss of both activities (X. Cecchi, unpublished work).

Iodination at alkaline pH modifies tyrosine residues (Simpson & Vallee, 1966) and tyrosinase may oxidize these residues in certain proteins, but none of these treatments is by itself specific for tyrosyl groups (Sizer, 1953). It seemed thus important to use other reagents that would specifically affect this amino acid.

Besides obtaining information about the participation of certain amino acid residues in the enzymic activity of apyrase, this would also be a first step to explore the similarities and differences between the isoenzymes of apyrase which exhibit physical as well as kinetic differences (Traverso-Cori *et al.*, 1970).

The present paper describes the results obtained by several inactivation techniques by using a highly purified preparation of an isoenzyme of apyrase, with a high ATPase/ADPase ratio, which was homogeneous both in the ultracentrifuge (Traverso-Cori *et al.*, 1970) and in gel electrophoresis, obtained from a single variety of potato.

Experimental

Materials

All chemicals were reagent grade. Special chemicals were obtained from the following sources: nucleotides, buffers, *N*-acetylimidazole, *p*-chloromercuribenzoate, 1-ethyl-3-dimethylaminopropylcarbodiimide, glycine methyl ester hydrochloride, 2,4,6-trinitrobenzenesulphonic acid, maleic anhydride,

* Abbreviations: ATPase, adenosine triphosphatase; ADPase, adenosine diphosphatase.

ribose, ribose 5-phosphate and serum albumin from Sigma Chemical Co., St. Louis, Mo., U.S.A.; tetranitromethane from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) from Cyclo Chemical Co., Los Angeles, Calif., U.S.A.; mushroom tyrosinase from Worthington Biochemical Co., N.Y., U.S.A.; monoiodoacetic acid from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. (used recrystallized from water three times).

Methods

Assay of apyrase. Apyrase activity was assayed as previously described (Traverso-Cori *et al.*, 1965) by measuring the liberation of P_i (Fiske & SubbaRow, 1925) from ATP or ADP.

Determination of protein. Approximate protein concentration was estimated from E_{280} (Warburg & Christian, 1941), a value of 1.0mg/ml per cm for E_{280} being assumed.

Apyrase purification. Apyrase was obtained from a homogeneous strain of *Solanum tuberosum* (var. Pimpernel) obtained by clonal selection, supplied by the Instituto de Investigaciones Agropecuarias 'La Platina', Santiago, Chile.

The enzyme was purified as previously described (Traverso-Cori *et al.*, 1970) with the following modifications. (a) A solution of 10mM-thioglycollic acid (Anderson, 1968) was used instead of water in the preparation of the extract and during the $(NH_4)_2SO_4$ fractionation, to avoid the browning due to tyrosinase activity. (b) The enzyme was incubated with 100mM-KCN for 2h at 0°C after the third $(NH_4)_2SO_4$ precipitation (Traverso-Cori *et al.*, 1970). Excess of KCN was removed by dialysis against 50mM-potassium succinate buffer, pH4.0, containing 300mM-KCl and 20mM-thioglycollic acid. This treatment, which removes copper from tyrosinase (Kubowitz, 1938), was repeated three times before the enzyme preparation was applied to the Sephadex G-25 column (Traverso-Cori *et al.*, 1970). This column was equilibrated with 50mM-potassium succinate, pH4.0–400mM-KCl–1mM-thioglycollic acid. (c) The filtration process was done as previously described (Traverso-Cori *et al.*, 1970), but the Sephadex G-150 column was replaced by Sephadex G-100. The next purification step was the passage through a CM-cellulose column (Traverso-Cori *et al.*, 1970). (d) The KCl gradient was omitted and the enzyme was eluted directly after the equilibration buffer with 1M-KCl. The fractions with apyrase activities were concentrated (Traverso-Cori *et al.*, 1970) to about 10mg of protein/ml. The enzyme was passed again through a CM-cellulose column and concentrated to 11.4mg of protein/ml under the same conditions. All the experiments described were done with the same final enzyme preparation with a specific activity of 600μmol of P_i released from ATP/min per mg.

Effect of pH on K_m and $V_{max.}$. The K_m and $V_{max.}$ values (Dixon & Webb, 1958) of apyrase at various pH values were determined at 30°C, employing the buffers described by Good *et al.* (1966) or β-alanine according to their pK. The final buffer concentration in all cases was 100mM. ATP and ADP ranged from 0.25 to 1.6mM. Ca^{2+} concentration was 5mM. Under these circumstances there was a large excess of metal (Nanninga, 1957). The appearance of P_i was measured (Ernster *et al.*, 1950) at two time-intervals. The total hydrolysis of substrates did not exceed 10% of the initial concentration. K_m and $V_{max.}$ were calculated from the results by the method of the least-squares with an Olivetti Programma 101 computer. No appreciable inactivation of the enzyme in the absence of substrates occurred even at the extreme pH values.

Ultracentrifugation. This was performed in a Spinco model E ultracentrifuge under the conditions described by Traverso-Cori *et al.* (1970) at different pH values, as described in the Results section.

Disc electrophoresis. The apyrase preparation (25μl) was subjected to the standard disc-electrophoresis technique. This was performed in a Canalco apparatus, model G (Bethesda, Md., U.S.A.) at pH8.3 (Davis, 1964) and pH4.3 (Reisfield *et al.*, 1962). The ATPase and ADPase activities were determined after elution of the enzyme from the gel (Traverso-Cori *et al.*, 1970).

Oxidation of apyrase by tyrosinase. Apyrase (0.5ml, 11.4mg of protein/ml) was placed in a dialysis bag attached to one end of an open glass tube. This bag was immersed in a beaker at 25°C, containing 100ml of 450mM-sodium acetate at pH4.0–4mM-pyrocatechol and 1760 units of tyrosinase (Sizer & Brindley, 1950), with continuous stirring. After 4h the apyrase preparation had become brown and samples were taken for the assay of ATPase and ADPase activities. Oxidized enzyme (25μl; 29μg) and an untreated control were subjected to disc electrophoresis at pH4.3.

Modifications of amino acid residues. (*a*) General procedures. All treatments of apyrase, if not otherwise indicated, were performed at 0°C with 0.45mg of protein/ml as time-course experiments. The reaction was stopped by diluting fivefold to 20-fold with serum albumin (0.20mg/ml). Controls were performed with solvents. It was also always established that the concentration of modifying reagent carried over to the assay medium did not interfere with the assay of apyrase activity.

When protection by substrates was explored control experiments were done to exclude a reaction of the substrate with the amino acid-modifying reagent, which could produce a significant decrease of the concentration of the latter. This was done by preincubating ATP or ADP with the amino acid reagent

for 1h before addition of the enzyme. These experiments were compared with results obtained without previous preincubation of the substrates with amino acid reagents. With reagents for amino groups, ITP or IDP were used as protecting substrates.

The optimum concentration of modifying reagent for the time-course experiments was established by using different concentrations of the reagent in single-time experiments.

(*b*) Iodination. Apyrase preparations were iodinated with 0.05mM-iodine at 20°C in 500mM-Tris–HCl buffer, pH8.0 (Traverso-Cori *et al.*, 1970). The formation of iodotyrosine was measured by E_{320} (Simpson & Vallee, 1966) after the reaction had been stopped by the addition of $Na_2S_2O_3$ to a final concentration of 6mM. The excess of reagents was removed by dialysis against 100mM-KCl.

(*c*) Acetylation. A freshly prepared solution of *N*-acetylimidazole (Riordan *et al.*, 1965) was added to a final concentration of 80mM to a solution of apyrase in 500mM-sodium borate buffer, pH7.5. The half-life of *N*-acetylimidazole in water at pH7.5 was 180min at 0°C and 13min at 30°C. The final concentration of *N*-acetylimidazole after a 4h experiment was estimated to be still great enough to acetylate the enzyme.

The formation of *O*-acetyltyrosine (Riordan *et al.*, 1965) was ascertained by the change in the absorption spectrum at 278nm. Reversal of the acetylation was performed with hydroxylamine as described by Domschke *et al.* (1970).

(*d*) Nitration. Treatment of apyrase with 2.5mM-tetranitromethane at pH8.0 and 20°C or 75mM-tetranitromethane at 0°C was done according to the method of Sokolovsky *et al.* (1966). At pH values below 7.0 no inactivation was observed (Sokolovsky *et al.*, 1966). E_{428} was measured (Sokolovsky *et al.*, 1966) at the same time-intervals as apyrase activity.

(*e*) Photo-oxidation. Photo-oxidation (Weil, 1965) was performed in 500mM-potassium succinate buffer, pH6.5, with a 100W projector lamp placed 8–12cm from the liquid surface, in the presence of 0.04% (w/v) Methylene Blue. Control experiments were performed in darkness.

(*f*) Alkylation. Apyrase solutions were allowed to react with 21mM-2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) (Barman & Koshland, 1967) in the dark in 500mM-sodium acetate buffer, pH4.0. The modified tryptophyl residue was measured by the E_{410}, pH7.0, or E_{320}, pH4.0, in the same sample by changing the pH (Barman & Koshland, 1967).

(*g*) Oxidation. Apyrase (0.27mg of protein/ml) at 0°C in 1M-$HClO_4$ adjusted to pH3.0 with NaOH was allowed to react with 88mM-H_2O_2 (Neumann *et al.*, 1962).

(*h*) Carboxymethylation. Protein (0.27mg/ml) was treated with 27mM-monoiodoacetic acid (Neumann *et al.*, 1962) in 1M-$HClO_4$ adjusted to pH3.0 with NaOH.

(*i*) Treatment with *p*-chloromercuribenzoate. The enzyme was allowed to react with 1mM-*p*-chloromercuribenzoate in the presence of 2M-urea for 4h (Bárány & Bárány, 1959).

(*j*) Carbodi-imide nucleophil procedure. The enzyme was allowed to react according to the procedure of Hoare & Koshland (1967).

Amino groups. 2,4,6-Trinitrobenzenesulphonic acid was used in the method of Freedman & Radda (1968); changes in extinction were observed at 340nm. The maleic anhydride technique was also used (Butler *et al.*, 1969).

Amino acid analysis. Analyses were performed by the method of Spackman *et al.* (1958) with Spinco models 120B and 120C automatic amino acid analysers.

Results and Discussion

Preparation of a high ATPase/ADPase ratio isoenzyme of apyrase

The use of potatoes of a single clonal variety (Pimpernel), which contain a large proportion of the isoenzyme with a high ATPase/ADPase ratio, has allowed us to prepare this particular form of apyrase with a specific activity of 600 units/mg of protein, which is two to six times higher than the purity previously obtained in this laboratory (Traverso-Cori *et al.*, 1965, 1970).

Apyrase with an ATPase/ADPase ratio of 10.0 was eluted from a CM-cellulose column as a single peak. The enzyme exhibited also a single anionic band both of protein and apyrase activity on polyacrylamide-gel electrophoresis at pH4.3 and 8.3. The ATPase/ADPase ratio was 10.0 in both cases. The mobility at pH8.3 was very low.

Fig. 1 shows the elution pattern of apyrase when the preparation was passed for a second time through a CM-cellulose column, appearing as a single peak with an ATPase/ADPase ratio of 10.0. The protein fraction emerging with the equilibration buffer had no enzymic activity, and there were no traces whatsoever of an apyrase with a low ATPase/ADPase ratio. The enzyme eluted from this column had the same specific activity as the peak eluted from the first CM-cellulose column, and it behaved on polyacrylamide gel in the same way as the enzyme eluted from the first CM-cellulose column. Both enzyme preparations migrated as a single peak in the ultracentrifuge, with a sedimentation coefficient of 3.0S, which did not vary between pH4.0 and 8.0.

There are marked differences between this apyrase from var. Pimpernel potatoes and the enzyme prepared previously from potatoes obtained from commercial sources. The latter contain measurable amounts of an isoenzyme of low ATPase/ADPase ratio, which may be separated on CM-cellulose

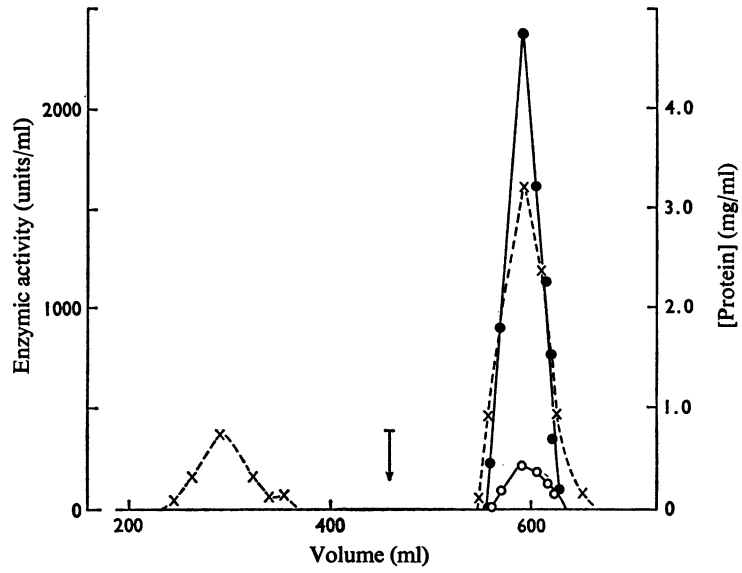

Fig. 1. *Chromatography on CM-cellulose of an isoenzyme of apyrase (ATPase/ADPase ratio = 10.0)*

Bed volume: 340ml; equilibration buffer: 50mM-potassium succinate, pH6.0. Protein (270mg; 162000 units of ATPase and 16200 units of ADPase) was applied and the column was further washed with buffer. Less than 10% of the protein and no enzyme activity was eluted by the buffer. ×, Protein; ●, ATPase activity; ○, ADPase activity. At the point indicated by the arrow, the equilibration solution was replaced by 1M-KCl solution in the same succinate buffer. The enzyme used for this experiment was previously chromatographed on the same type of CM-cellulose column under the same conditions. The fractions collected between 550 and 650ml, containing the highest apyrase activities, were pooled and concentrated to 4.5ml and then applied to the column described in this figure. The elution pattern was the same. The specific activity and ATPase/ADPase ratio of all the fractions of the enzyme peak were constant.

(Traverso-Cori *et al.*, 1970, Fig. 4), whereas the former do not contain any traces of the low ATPase/ADPase ratio isoenzyme at this stage of purification (Fig. 1). This ensures the absence of that isoenzyme from the preparation used in the present report.

Stability of the enzyme, on storage at 4°C, and yield were also improved above those of previously published procedures (Traverso-Cori *et al.*, 1965, 1970).

Effects of tyrosinase on apyrase

The most significant improvement in the purification of apyrase was the suppression of tyrosinase activity present in potatoes. This was achieved by the treatment of the enzyme preparation with thioglycollic acid (Anderson, 1968) and KCN (Kubowitz, 1938) (see the Experimental section).

Treatment of an electrophoretically homogeneous apyrase preparation with tyrosinase and catechol produced two new bands of enzymically inactive protein. Ion-exchange chromatography of the oxidized enzyme preparation on CM-cellulose shows an increase of the amount of inactive protein eluted from the column at low ionic strength (Fig. 1). This shows that apyrase inactivation by oxidation is concomitant with a change in physicochemical properties. The total enzymic activity decreased by 55% for ATPase and 25% for ADPase after this treatment. These effects were due to the action of the oxidation products of catechol, and not to a direct effect of tyrosinase, since the two proteins were separated by a dialysis membrane (Sizer & Brindley, 1950). Further, apyrase was not affected under the same experimental conditions, if catechol was omitted from the dialysis medium, or if the two enzymes were mixed in the absence of catechols.

The inactivation by the reaction products of tyrosinase is suggestive of the participation of tyrosyl groups in apyrase activity. However, since this is not a specific reaction (Sizer, 1953) the results may be attributed to the participation of other amino acids instead of or in addition to tyrosyl residues.

K_m and $V_{max.}$ at different pH values

When potato apyrase was assayed at different pH values and substrate concentrations, the profile of the log $V_{max.}$ versus pH curve showed only one significant inflexion about pH4.7 for ATPase activity and pH5.2 for ADPase activity (Fig. 2). This difference of 0.5pH unit could be accounted for by the difference of pK values of Ca^{2+}–ATP and Ca^{2+}–ADP, which are 5.13 and 5.58 respectively (Nanninga, 1957).

The plot of pK_m as a function of pH shows clear negative inflexions for both activities at pH4.1, which could indicate the participation of carboxyl groups (Dixon & Webb, 1958). The binding of the substrates could conceivably change the charge of the protein to the pK values observed in the $V_{max.}$ curve.

The amino acids at the active site for ATP could have pK values different from those at the ADP site, as suggested by the widely different pattern of inflexions of the two pK_m curves.

Effects of modifiers of the tyrosyl residue

Table 1 shows that iodination at pH8.0 produced a decrease of both enzymic activities of apyrase, and that substrates did not protect the enzyme from inactivation. Changes in extinction between 300 and 360nm, with a maximum increase at 320nm, were observed. This spectrum is characteristic for the iodination of tyrosine (Simpson & Vallee, 1966).

Treatment of the enzyme with *N*-acetylimidazole produced a change in the absorption spectrum

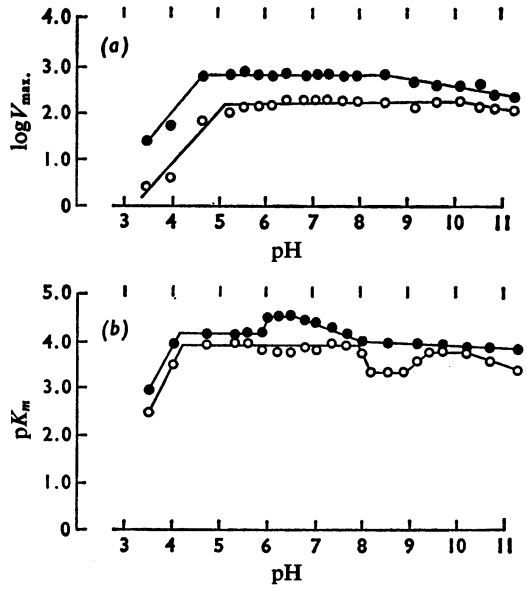

Fig. 2. *Dixon plots of apyrase*

(*a*) log $V_{max.}$ versus pH of ATPase (●) and ADPase (○) activity of apyrase. (*b*) Negative log K_m versus pH of ATPase (●) and ADPase (○) activity of apyrase. The standard deviation for $1/V_{max.}$ and $-1/K_m$ of the primary Lineweaver–Burk graphs ranged between 1.0 and 5.5%.

Table 1. *Effects of modifiers of the tyrosyl residues on apyrase activity*

Apyrase was treated with protein reagents at the concentrations indicated, as described in the Experimental section, either with or without further additions of the protecting agents specified. After 1 min for iodine or 60min for *N*-acetylimidazole or tetranitromethane, the reaction was stopped and the enzyme was assayed with ATP or ADP. For further details see the Experimental section.

			Inactivation (%)					
			ATPase			ADPase		
Reagent	Concn. (mM)	pH	No addition	Protecting agent added (10mM)		No addition	Protecting agent added (10mM)	
Iodine	0.2	8.0	67	ATP	67	44	ATP	44
				ADP	67		ADP	44
Acetylimidazole	80.0	7.5	82	ATP	23	42	ATP	13
				ADP	49		ADP	20
Tetranitromethane	75.0	8.0	68	ATP	23	38	ATP	13
				ADP	49		ADP	20
				AMP	40		AMP	23
				PP_i	46		PP_i	24
				Ribose 5-*P*	64		Ribose 5-*P*	30
				Ribose	70		Ribose	29

Table 2. *Recovery of apyrase activities from acetylated enzyme by neutral hydroxylamine*

Two samples of apyrase acetylated as detailed in the Experimental section and one of untreated enzyme were adjusted to pH2.2 and incubated for 10min at 20°C. After this, pH was readjusted to 7.0, and to samples (*a*) and (*b*) neutral hydroxylamine was added to a final concentration of 1.0M. Incubation at 20°C was continued and samples for enzymic assay were taken at the indicated time-intervals. The results are expressed as % of enzymic activity referred to the non-acetylated enzyme.

	Enzymic activity (%)					
Incubation time (h) ...	1		24		48	
	ATPase	ADPase	ATPase	ADPase	ATPase	ADPase
(*a*) Non-acetylated enzyme (treated with 1.0M-hydroxylamine)	100	100	100	100	100	100
(*b*) Acetylated enzyme (treated with 1.0M-hydroxylamine)	9.6	44.0	18.3	74.5	31.0	77.0
(*c*) Acetylated enzyme (without hydroxylamine)	7.1	27.0	7.8	29.0	11.4	32.5

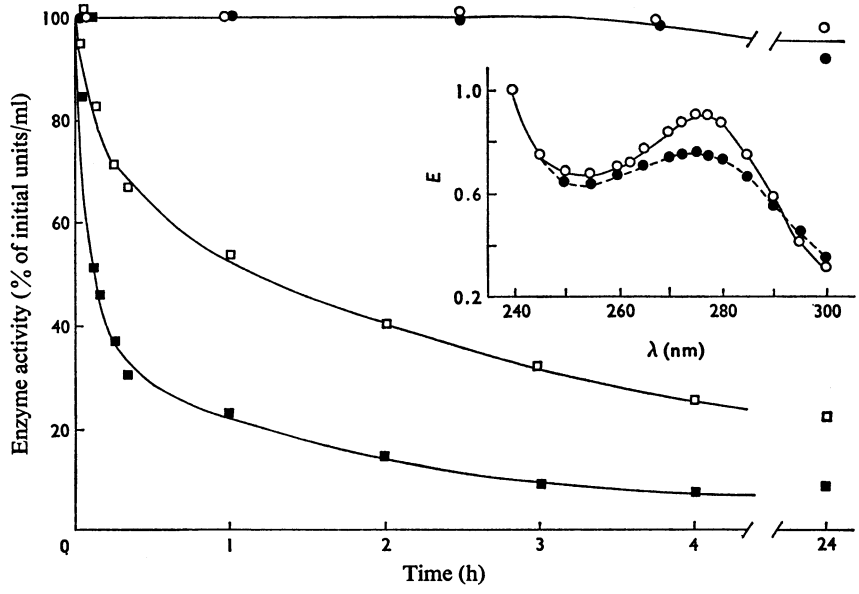

Fig. 3. *Acetylation of apyrase* (0.45*mg of protein/ml*) *with* 80*mM-N-acetylimidazole at pH*7.5 *and* 0°*C*

■, ATPase, acetylated; □, ADPase, acetylated; ●, ATPase, control; ○, ADPase, control. The inset shows the absorption spectra of the acetylated (●---●) and non-acetylated (○——○) apyrase after 24h of treatment by previous dialysis (see Experimental section). These spectra show a characteristic difference of extinction at 278nm (Riordan *et al.*, 1965).

(Fig. 3), which has been described (Riordan *et al.*, 1965) as characteristic of *O*-acetylation of tyrosine, with a maximum change in extinction at 278nm. Both enzymic activities decreased, in an approximately parallel fashion, but the initial rate of decrease was higher for the ATPase activity. Both substrates protected both activities (Table 1).

Table 2 shows that treatment of the acetylated enzyme with hydroxylamine slowly restored both activities, although not completely. The loss of ATPase activity was reversed to a lesser extent than that of ADPase activity. *N*-Acetylimidazole acetylates phenolic hydroxyl groups and to a lesser extent lysyl groups of proteins (Riordan *et al.*, 1965). Treatment

with hydroxylamine at neutral pH cleaves the phenolic ester bond, allowing restoration of enzyme activity, but it does not affect the *N*-acetyl bond (Domschke *et al.*, 1970). This may be the rationale for the different percentages of restoration observed for ATPase and ADPase activities by treatment with hydroxylamine (Domschke *et al.*, 1970).

Nitration with tetranitromethane, under the experimental conditions used, has been reported to be very specific for tyrosyl residues (Sokolovsky *et al.*, 1966).

Fig. 4 shows that both activities were affected by nitration, and that increase of E_{428} mirrored the decrease of activity.

Analysis of 24h or 48h acid hydrolysates of native and nitrated apyrase showed the appearance of 0.023 μmol of nitrotyrosine/mg of protein used. There was a concomitant disappearance of tyrosyl residues. The small amount of sample used did not allow us to match these two quantities (Vincent *et al.*, 1970), but the appearance of a nitrotyrosyl peak is of qualitative importance.

Substrate protection of apyrase from nitration suggests that the amino acid residue attacked by tetranitromethane reacts with the substrate or is covered

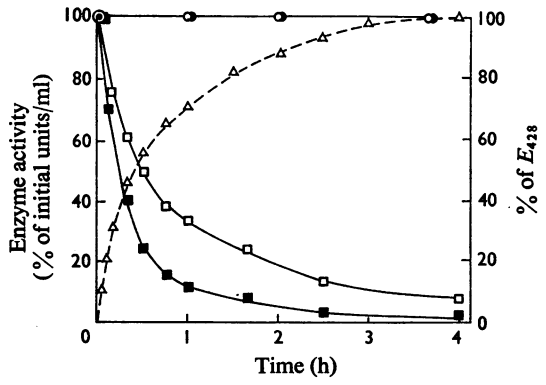

Fig. 4. *Nitration of apyrase* (0.45 *mg of protein/ml*) *with* 2.5 mM-*tetranitromethane at pH* 8.0 *and* 20°C

■, ATPase, nitrated; □, ADPase, nitrated; ●, ATPase, control; ○, ADPase, control. △– –△, % of extinction at 428nm of the modified tyrosyl residue of apyrase, which was measured simultaneously with the measurement of both enzyme activities (Sokolovsky *et al.*, 1966).

Table 3. *Effect of modifying reagents on apyrase activity*

Apyrase was treated with the protein reagents at the concentration indicated, as described in the Experimental section, either with or without further additions of the protecting agents specified. After 60 min incubation the reaction was stopped and the enzyme was assayed with ATP or ADP. Further details are given in the Experimental section.

			Inactivation (%)				
			ATPase			ADPase	
Reagent	Concn. (mM)	pH	No addition	Protecting agent added (10mM)		No addition	Protecting agent added (10mM)
Methylene Blue (photo-oxidation)	1.25	6.5	45	ATP	45	25	ATP 25
				ADP	45		ADP 25
H_2O_2	88.0	3.0	25	ATP	25	12	ATP 12
				ADP	25		ADP 12
Koshland's reagent	21.0	4.0	65	ATP	17	56	ATP 30
				ADP	35		ADP 15
Monoiodoacetic acid	27.0	3.0	78	ATP	47	72	ATP 45
				ADP	41		ADP 46
Carbodi-imide	40	4.75	70	ATP	70	55	ATP 55
				ADP	70		ADP 55
2,4,6-Trinitrobenzenesulphonic acid	30	8.0	60	ITP	38	25	ITP 9
				IDP	35		IDP 9
Maleic anhydride	100	8.0	88	ITP	79	58	ITP 28
				IDP	79		IDP 29
p-Chloromercuribenzoate	1.0	4.0	0	—		0	—
p-Chloromercuribenzoate+2M-urea	1.0	4.0	0	—		0	—

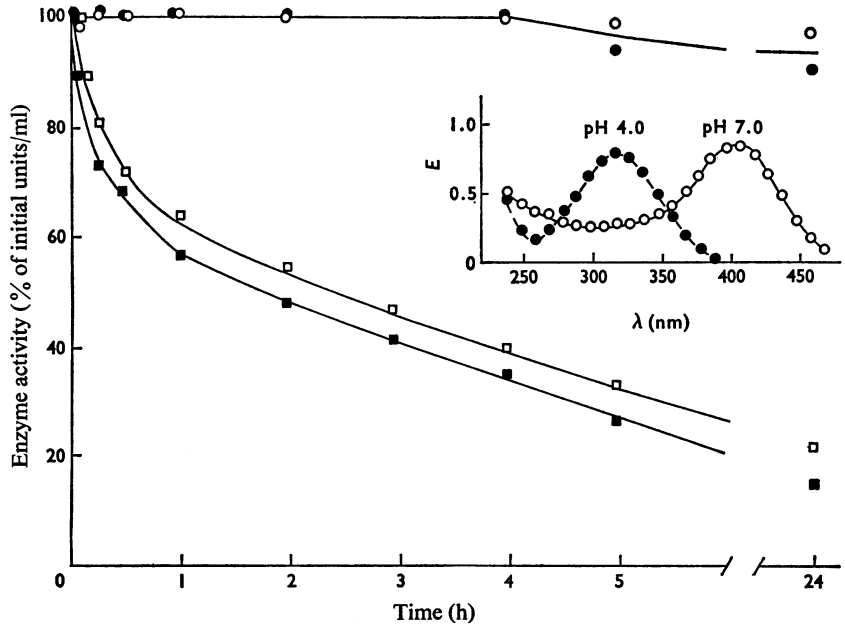

Fig. 5. *Alkylation of apyrase* (0.45 *mg of protein/ml*) *in the dark at pH* 4.0, 0°*C, with* 21 *mM-2-hydroxy-5-nitrobenzyl bromide*

■, ATPase, alkylated; □, ADPase, alkylated; ●, ATPase, control; ○, ADPase, control. The inset shows the absorption spectra at pH 4.0 and pH 7.0 of the same sample of the alkylated apyrase at the end of 24h incubation, after previous dialysis (for more details see the Experimental section).

in the conformational changes of the enzyme induced by the substrate. The fact that some inhibitors, like AMP and PP_i (Cori *et al.*, 1965), also have a certain degree of protection for ATPase and ADPase activities strengthens this assumption. However, substances like ribose 5-phosphate or ribose, which are neither substrates nor inhibitors, did not show any appreciable protection.

ATPase activity decayed faster than ADPase activity on treatment with most of the tyrosyl residue reagents, including enzymic oxidation by oxidized catechols generated by tyrosinase (Sizer & Brindley, 1950). The difference in the rate of inactivation of the ATPase and ADPase activities suggests the participation of two or more tyrosyl residues with different reactivities toward tyrosine-modifying reagents. However, there are other ways of interpreting this observation, such as the modification of a single tyrosine residue having a differential effect on the two activities.

Treatment with tetranitromethane at pH 8.0 modifies tyrosyl and cysteinyl residues (Sokolovsky *et al.*, 1966). The latter may be ruled out by the insensitivity of apyrase to *p*-chloromercuribenzoate (Table 3), both in the presence and in the absence of urea. The effect of monoiodoacetic acid on apyrase is independent of pH (Cori *et al.*, 1965), which also speaks against the participation of thiol groups (Bárány & Bárány, 1959). These experiments point very strongly to the participation of a tyrosyl residue in both activities of this particular isoenzyme of apyrase.

Effects of other amino acid modifiers

The pattern of inactivation by different amino acid reagents and the protection by substrates shown in Table 3 may be interpreted as being due to modification of histidyl (Weil, 1965), methionyl (Neumann *et al.*, 1962) and tryptophyl residues (Barman & Koshland, 1967).

The participation of carboxylic groups, which is suggested by the pH profiles and by inactivation by carbodi-imide (Hoare & Koshland, 1967), is not likely to occur near the active site, since there was no observable protection by substrates.

Koshland's alkylating reagent has been reported to be very specific for tryptophyl residues (Barman & Koshland, 1967). Fig. 5 shows the parallel decay of both enzymic activities and changes in extinction at 320 and 410nm, which are the maxima reported

for alkylated tryptophan at pH4.0 and 7.0 respectively.

The inactivation of apyrase by oxidation with H_2O_2 and carboxymethylation with iodoacetic acid in acid conditions points to the participation of methionyl groups. Both activities of apyrase were modified at the same rate by carboxymethylation, which suggests the participation of methionyl groups at the active site (Gurd, 1967). Loss of activity by oxidation with H_2O_2 also points to the participation of the same amino acid since oxidation of tryptophanyl residues is much slower than that of methionyl groups at acid pH (Neumann, 1967).

The participation of amino groups seems doubtful, in spite of the fact that there was inactivation by 2,4,6-trinitrobenzenesulphonic acid and protection by substrate. However, changes of E_{340} were much faster than inactivation of the enzyme. Maleylation of amino groups produced an irreversible inactivation of both enzymic activities (Butler *et al.*, 1969).

It may be concluded that tyrosyl and tryptophyl residues are necessary for both enzymic activities of this isoenzyme of apyrase with a high ATPase/ADPase ratio. These two activities could involve distinct tyrosyl residues, thus accounting for the different rates of inactivation. The participation of methionyl residues seems less certain. Most of the reagents used modify histidyl residues, but since other residues may be modified concomitantly, the participation of this group cannot be ascertained at this moment.

The earlier view (Cori *et al.*, 1965; Traverso-Cori *et al.*, 1965, 1970) that both ATPase and ADPase activities are present in a single enzyme molecule is thus complemented by the hypothesis that the substrate sites may be somewhat different, as suggested by the different rates of loss of ATPase and ADPase activities when tyrosyl residues were modified. Some other residues, like tryptophyl and perhaps methionyl, could be shared by the two active sites, thus explaining the identical course of inactivation.

It will be of interest to obtain an isoenzyme of low ATPase/ADPase ratio of comparable purity, to explore the effects of amino acid reagents on both types of apyrase, which had been suggested to be different in an earlier communication.

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