Biochimica et Biophysica Acta, 660 (1981) 325–332
Elsevier/North-Holland Biomedical Press

BBA 69321

CHLOROPLAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (NADP$^+$)

REACTIVITY OF ESSENTIAL CYSTEINE RESIDUES IN HOLO- AND APOENZYME

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(Received December 29th, 1980)

Key words Glyceraldehyde-3 phosphate dehydrogenase (NADP$^+$), Cysteine residue, (Chloroplast)

On treatment with iodoacetic acid both native and apo-glyceraldehyde-phosphate dehydrogenase (NADP$^+$) (D-glyceraldehyde-3-phosphate NADP$^+$ oxidoreductase (phosphorylating), EC 1 2 1 13) from spinach chloroplasts are inactivated following a pseudo-first-order process The rate of inactivation as well as the affinity for the reagent are lower in apo- than in holoenzyme NADP is more effective than NAD in raising the reaction rate of apoprotein with iodoacetic acid and also enhances the rate of inactivation of native enzyme Although kinetic evidence indicates that both in native and apo-glyceraldehyde-phosphate dehydrogenase (NADP$^+$) the loss of activity depends on the reaction of 1 mol iodoacetic acid per active site, a different extent of labelling has been found in the proteins two and four carboxymethyl residues are incorporated per protomer (80 000 molecular weight) in completely inactivated holo- and apoenzyme, respectively The reactive cysteine residues are located in different tryptic peptides, as suggested by the finding of two labelled spots on fingerprints of holoenzyme in addition to these, two other faintly radioactive spots appear in inactivated apoenzyme Partially inactivated apoprotein has been separated by affinity chromatography into a completely inactive species and a fully active one the relative amount of these species depends on the extent of inactivation of the sample The number of alkylated cysteines in the fully inactive apoprotein is found to vary as a function of the inactivation extent, and a minimum of two residues per M_r 80 000 has been found The two different subunits making up the enzyme (43 000 and 37 000 daltons, respectively) behave asymmetrically during the alkylation process showing that the reaction between glyceraldehyde-phosphate dehydrogenase (NADP$^+$) and iodoacetic acid takes place first at the 37 000-dalton subunit

Introduction

The main form of glyceraldehyde-3-phosphate dehydrogenase (NADP$^+$) from spinach leaf chloroplasts (D-glyceraldehyde-3-phosphate NADP$^+$ oxidoreductase (phosphorylating), EC 1 2 1 13) is a multimeric protein of 600 000 daltons, active with either NADP(H) or NAD(H) as coenzymes [1–8] Unlike the NAD-linked glyceraldehyde-phosphate dehydrogenases, which are tetramers of chemically identical subunits [7,9–13] the chloroplast enzyme is thought to result from the assembly of 80 000 dalton protomers [6], each made up of two non-identical subunits of 43 000 and 37 000 daltons, respectively [3,6–8] *

Native enzyme has been found to bind 4 mol NADP and 4 mol NAD per 600 000 daltons the removal of bound pyridine nucleotides results in an apoprotein which still retains the same kinetic properties but, unlike the holoenzyme, gradually loses its activity [6]

Pyridine nucleotides have been reported to affect,

* We indicate as subunit of the enzyme any single peptide chain and by protomer (80 000 daltons) the association of a 43 000 and a 37 000-dalton subunit

in vitro, the aggregation states as well as the catalytic properties of the protein [2,3,4,7,14] A reversible dissociation (from 600 000 to 145 000 daltons) which is accompanied by an enhancement in NADP(H)-linked activity has been described upon addition of NADP or NADPH, on the contrary NAD has seen to produce aggregation of the protein [2,4,7]

Because of such peculiar features, the chloroplast enzyme offers a structural and functional model which appears to differ markedly from that of glyceraldehyde-phosphate dehydrogenases so far known it seemed, therefore, interesting to obtain further information on this enzyme and to compare its properties with those of the counterparts from other sources

In this paper we investigated the reactivity of native and coenzyme-free glyceraldehyde-phosphate dehydrogenase from spinach leaf chloroplasts towards iodoacetic acid the main results reported here show, together with a conformational change induced by pyridine nucleotides, an asymmetric behaviour of the two subunits during the alkylation process

Materials and Methods

Glyceraldehyde 3-phosphate, diethylacetal barium salt, β-$NADP^+$, β-NAD^+ were crystalline preparations from Sigma Chemical Co (St Louis, MO, U S A) Iodoacetic acid was obtained from Fluka A G (Buchs, Switzerland) and recrystallized three times from ethyl ether and petroleum ether Iodo[$^{14}C_2$]-acetic acid was supplied by The Radiochemical Center (Amersham) Agarose hexane nicotinamide adenine dinucleotide phosphate type III was obtained from P L Biochemical Inc (Milwaukee, WI, U S A)

Tos-PheCH_2Cl-treated trypsin was supplied by Worthington Biochemical Co (NJ, U S A) All other chemicals were of analytical grade and were purchased from C Erba (Milan, Italy)

Enzyme purification and assay procedures The enzyme was prepared from fresh spinach leaves and assayed for the oxidative activity as previously described [6] Unless otherwise specified, the data reported in the table and figures refer to NADP-dependent activity

Apoprotein was obtained by charcoal treatment according to Cseke and Boross [15] Protein concentration was determined according to Lowry et al [16]

Treatment of the enzyme with iodoacetic acid Samples of holo- or apoenzyme (2 mg/ml, 25 μM assuming a molecular weight of 80 000) were incubated at 4°C in 45 mM pyrophosphate/5 mM EDTA buffer (pH 8 5) with different amounts of iodoacetic acid At different times, aliquots of 10 μl were diluted to 0 5 ml in pyrophosphate/EDTA buffer and activity assayed on 10 μl (0 4 μg protein)

Labelling with iodo[$^{14}C_2$]acetic acid (specific activity 1 μCi/μmol) was carried out by incubating holo- or apoenzyme in the above conditions After assay of activity, protein was precipitated with 4 vol cold acetone containing 5% 1 N HCl, and washed three times with the same solution The alkylated cysteine residues were determined by standard amino acid analysis and radioactivity measurement

Amino acid analysis, tryptic digestion and peptide maps These were carried out as previously reported [17]

Affinity chromatography It was performed on agarose/hexane/nicotinamide adenine dinucleotide phosphate column (1 × 2 5 cm, 2 ml gel bed) equilibrated with 45 mM sodium pyrophosphate/5 mM EDTA buffer (pH 8 5) The elution was carried out with 12 ml sodium pyrophosphate/EDTA buffer (pH 8 5), followed by 12 ml 10 mM NADP in the same buffer (1-ml fractions)

Polyacrylamide gel electrophoresis Electrophoresis on polyacrylamide gradient gel slabs was performed at pH 8 0 according to Wright et al [18], using the following reference proteins thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to Weber et al [19] For scintillation counting, solubilized gel was obtained by using *N,N'*-diallyltartardiamide as cross-linking agent [20]

Results

Inactivation of chloroplast glyceraldehyde-3-phosphate dehydrogenase

Treatment of native enzyme with iodoacetic acid leads to the inactivation of both NADP- and NAD-dependent activities following a pseudo-first-order process Fig 1A shows the loss of NADP-dependent

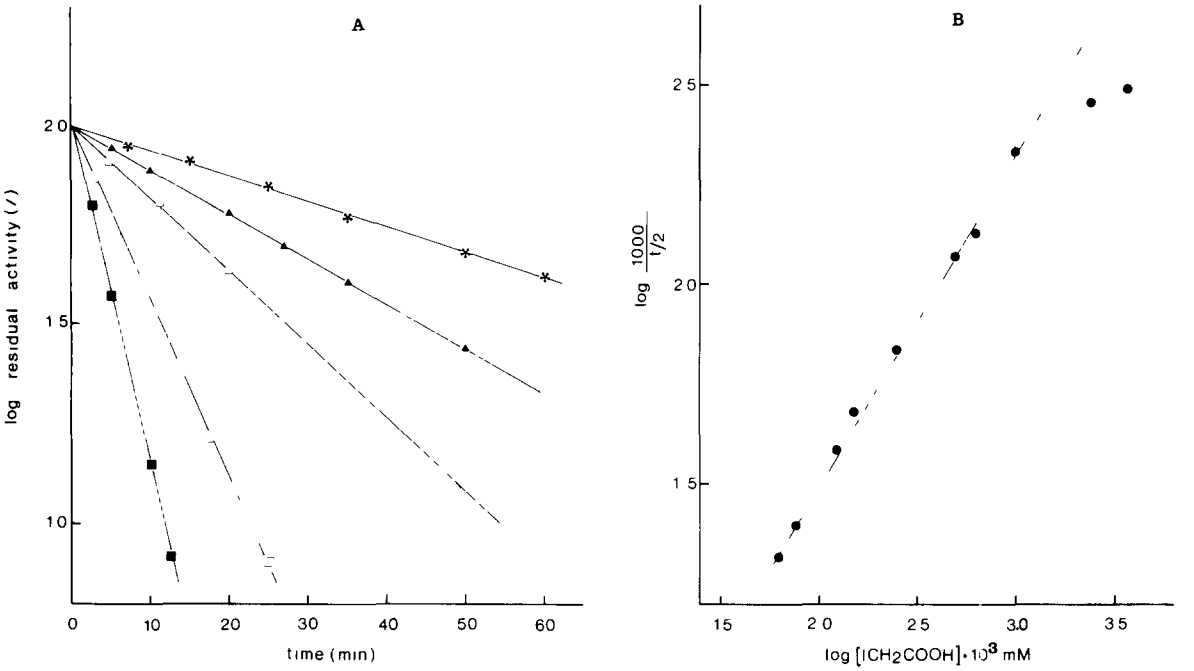

Fig 1 A Time course of the inactivation of holo-glyceraldehyde-phosphate dehydrogenase by iodoacetic acid Native enzyme (25 μM assuming a molecular weight of 80 000) was treated with iodoacetic acid and assayed at the indicated times Iodoacetic acid concentrations *———*, 0 0625 mM, ▲———▲, 0 125 mM, △———△, 0 25 mM, □———□, 0 625 mM and ■———■, 2 5 mM B Order of the inactivation process of holo-glyceraldehyde-phosphate dehydrogenase with respect to iodoacetic acid concentration $t_{1/2}$ values were calculated from time course experiments of inactivation of holoenzyme at the indicated iodoacetic acid concentrations

activity similar patterns were obtained when NAD-dependent activity was assayed

The inactivation is complete after 1 mol reagent is bound per active site, as judged from the slope – 0 85 – of the plot of Fig 1B [21] A K_{inact} of 0 9 mM and a first-order rate constant of 3 85 10^{-3} s^{-1} can be calculated by replotting the data from Fig 1B according to Meloche [22]

Apo-glyceraldehyde-phosphate dehydrogenase (adsorbance ratio of 2 0 at 280/260 nm) was used immediately after preparation by charcoal treatment of native enzyme Its electrophoretic behaviour is indistinguishable from that of native enzyme, but unlike holoenzyme, it loses its activity, in pyrophosphate/EDTA buffer (pH 8 5) at 4°C with a $t_{1/2}$ of 29 h

The data of Fig 2A, corrected for spontaneous inactivation, show that the inactivation of apo-glyceraldehyde-phosphate dehydrogenase is also a pseudo-first-order process with a K_{inact} of 9 mM and a first-order rate constant of 1 05 10^{-3} s^{-1} its dependence on the binding of 1 mol reagent per active site is proved by the slope – 0 83 – of the plot reported in Fig 2B

In order to evaluate the relative contribution of NADP and NAD to the reactivity of glyceraldehyde-phosphate dehydrogenase, samples of apoprotein were incubated with different amounts of either pyridine coenzymes, then the rate of inactivation by identical amount of iodoacetic acid was measured The results reported in Table I show that maximal rate of inactivation is obtained at a molar ratio NADP apoprotein (protomer) of 2 1, NADP is also found to enhance the rate of inactivation of the native enzyme On the contrary NAD is shown to produce a less marked effect on apoprotein and no effect at all on holoenzyme inactivation

Modification of cysteine residues The data of Fig 3A demonstrate that the loss of activity is consistent with the labelling of two cysteine residues per

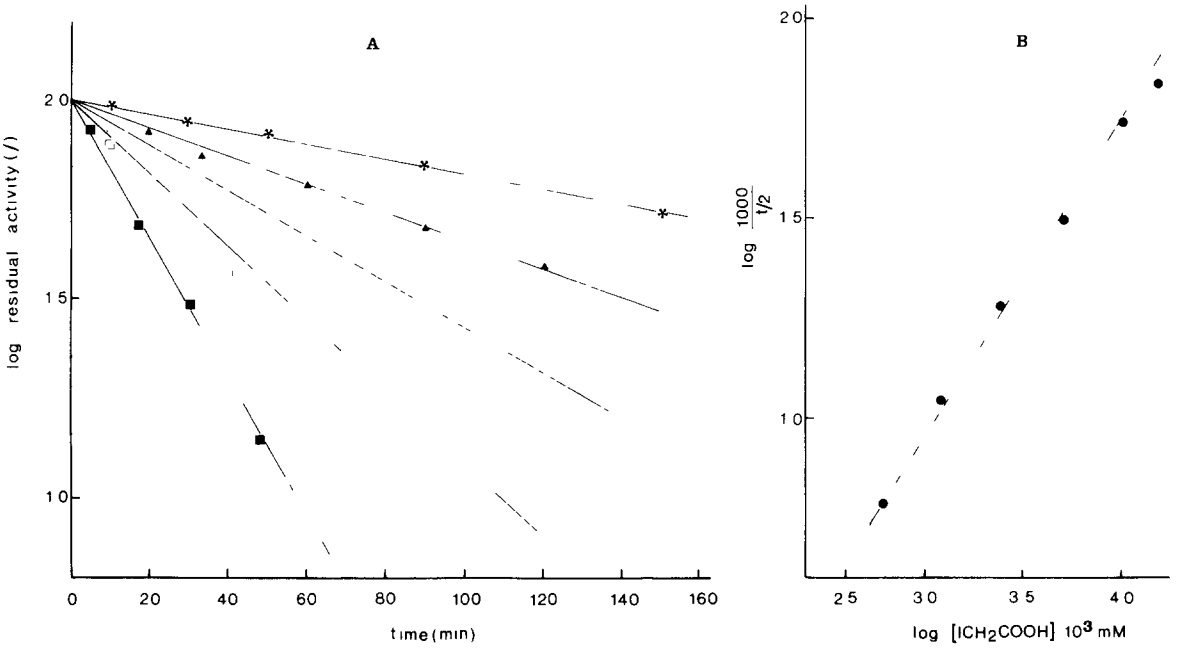

Fig 2 A Time course of the inactivation of apo-glyceraldehyde-phosphate dehydrogenase by iodoacetic acid For experimental details see Fig 1A Iodoacetic acid concentration *———*, 0 625 mM, ▲———▲, 1 25 mM, △———△, 2 5 mM, □———□, 5 mM and ■———■, 10 mM B Order of the inactivation process of apo-glyceraldehyde-phosphate dehydrogenase with respect to iodoacetic acid concentration $t_{1/2}$ values were calculated from time course experiments of inactivation of apo-enzyme at the indicated iodoacetic acid concentrations

TABLE I

EFFECT OF ADDED PYRIDINE COENZYMES ON THE RATE OF INACTIVATION OF HOLO- AND APO-GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE BY IODOACETIC ACID

Samples of protein (2 mg/ml, 25 μM assuming a molecular weight of 80 000) were incubated at 4°C in 45 mM pyrophosphate/5 mM EDTA buffer, pH 8 5 with pyridine coenzymes in the indicated molar ratios After 5 min, iodoacetic acid (final concentration 0 625 mM) was added at different times aliquots of 10-μl were taken from the incubation mixture and assayed for activity From each set of data $t_{1/2}$ was calculated

Molar ratios added coenzyme protomer (80 000 molecular weight)	$t_{1/2}$ (min)			
	Apoenzyme		Native enzyme	
	+NADP	+NAD	+NADP	+NAD
–	160 0	160 0	7 0	7 0
0 5 1 0	10 0	19 0	–	–
1 0 1 0	6 0	14 0	3 5	–
2 0 1 0	3 5	11 0	3 5	–
10 0 1 0	3 5	7 5	3 5	7 0

protomer in native enzyme, and four in apoprotein, respectively The plot of the same data according to Tsou [23] suggests that in both cases such a loss is due to the reaction of two residues, since the sets of data reported in Fig 3B allow us to conclude that the best fit is obtained for $t = 2$

The fact that the removal of bound pyridine coenzymes causes the exposure of other cysteine residues, in addition to those which are alkylated in holoenzyme, is proved by the analysis of tryptic peptide maps Fig 4 shows that four labelled peptides are detected in a tryptic peptide map of inactivated apoenzyme the order of reactivity as judged by visual comparison of the relative intensity on autoradiographic plates can be tentatively established to be peptide $a \geqslant b \gg c \simeq d$ Only two of these (peptides a and b) appeared on peptide maps of inactivated holo-glyceraldehyde-phosphate dehydrogenase, and the relative intensity of the radioactivity was the same as in the corresponding ones found in apoenzyme ($a \geqslant b$)

Tryptic maps were also carried out at different

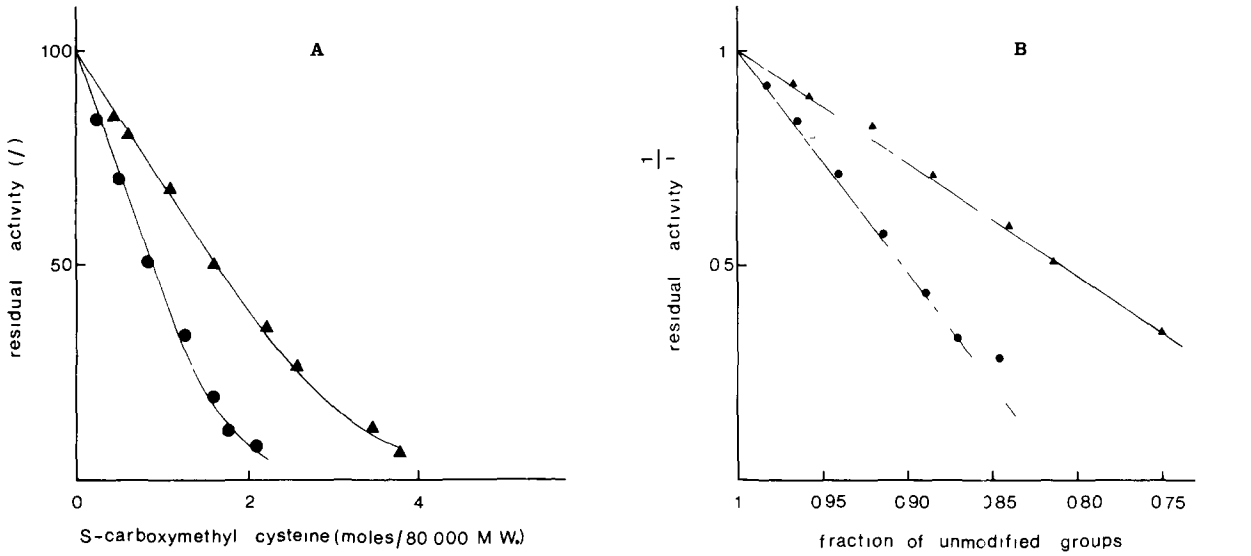

Fig 3 A Correlation between the number of alkylated cysteine residues and inactivation of holo- and apo-glyceraldehyde-phosphate dehydrogenase The data are the mean of five separate determinations from two sets of experiments carried out either at constant times and different iodoacetic acid concentrations (15 min and [ICH_2COOH] from 0 0625 to 2 5 mM for native enzyme, 20 min and [ICH_2COOH] from 0 625 to 10 mM for apoenzyme), or at constant iodoacetic acid concentration and different times (holoenzyme [ICH_2COOH] 0 625 mM from 3 to 25 min, apoenzyme [ICH_2COOH] 10 mM from 3 to 60 min) ●——●, holoenzyme, ▲——▲, apoenzyme B Replot of the data according to Tsou [23] Holoenzyme ○, $t = 1$, ●, $t = 2$ Apoenzyme △, $t = 1$, ▲, $t = 2$

times during the alkylation of either holo- or apo-protein the extent of labelling is found to increase progressively with the time of incubation but the patterns of the labelled tryptic peptides appear to be unmodified even at 75% residual activity two

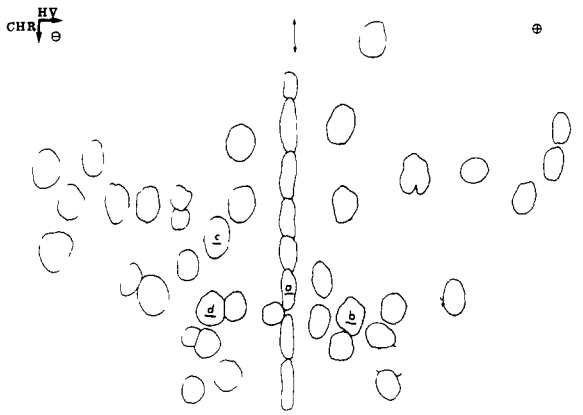

Fig 4 Tryptic peptide map of 90% inactivated apo-glyceraldehyde-phosphate dehydrogenase treated with iodo[$^{14}C_2$]-acetic acid a, b, c and d indicate radioactive peptides Dotted lines encircle faintly, stained spots

radioactive spots (a and b) and four radioactive spots (a, b, c and d) appear in holo- or in apoenzyme, respectively

In order to get additional information on the mechanism of inactivation, samples of apo-glyceraldehyde-phosphate dehydrogenase, at different stages inactivation, were submitted to affinity chromatography on Sepharose/hexane/NADP The elution patterns (Fig 5A and B) show that while the untreated apo-glyceraldehyde-phosphate dehydrogenase is completely retained and can be eluted only after the addition of coenzyme (10 mM NADP), partially inactivated apoenzyme travels as two species one, which is eluted with the solvent front, is completely inactive and retains most of the incorporated radioactivity, the second, eluted after addition of coenzyme, is still active and only barely labelled

The relative amounts of the protein eluted in each of the two peaks is dependent on degree of inactivation the content of *S*-carboxymethylcysteine of the species separated in the first peak is no less than 2 mol per protomer, however low the extent of the

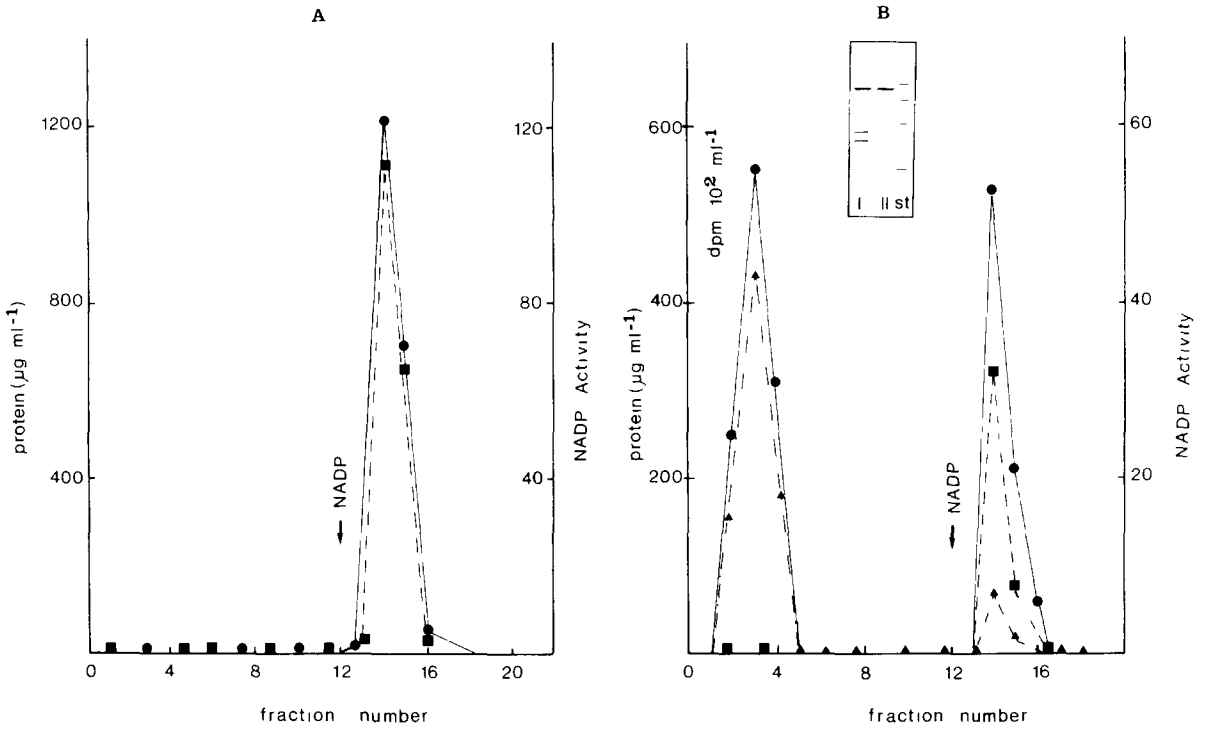

Fig 5 Affinity chromatography of 2 mg untreated (A) and partially inactivated (45% residual activity) apo-glyceraldehyde-phosphate dehydrogenase (B) on agarose-hexane NADP •, protein concentration (µg ml^{-1}) ■, NADP activity (µmol NADP reduced per min^{-1} ml^{-1}) ▲, radioactivity (dpm ml^{-1}) Inset of Fig 5B Gradient polyacrylamide gel electrophoresis of protein recovered from peaks I and II Standard proteins Thyroglobulin, ferritin, catalase, lactate dehydrogenase and albumin

alkylation is (Table II)

The carboxymethyl groups associated with the species eluted in the second peak have been found to be less than 0 5 per protomer

Carboxymethyl apoprotein obtained from the first peak gives, on polyacrylamide gel electrophoresis, several bands with molecular weights from 600 000 down to 145 000 daltons, an unique band of

TABLE II

AFFINITY CHROMATOGRAPHY OF PARTIALLY INACTIVATED APO-GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE

Samples of protein (2 mg/ml, 25 µM assuming a molecular weight of 80 000) were incubated at 4°C with iodo[$^{14}C_2$]acetic acid (10 mM) for different times (10, 20, 35 and 60 min) After gel filtration, each sample was submitted to affinity chromatography

Residual activity (%)	Recovered protein (%)		Recovered activity (%)		Alkylated cysteine residues (per 80 000 molecular weight)	
	peak I	peak II	peak I	peak II	peak I	peak II
60	39	61	0	98	2 00	0 40
45	60	40	0	99	2 50	0 41
26	75	25	0	96	2 90	0 44
10	90	9	0	97	3 60	0 46

600 000 molecular weight is evidenced in active apoprotein recovered from the second peak (inset of Fig 5B)

Both proteins behave as the untreated enzyme when submitted to disc-gel electrophoresis in denaturing media, showing that their subunit composition was unmodified

Reactivity of the subunits towards iodo[$^{14}C_2$]acetic acid

In order to detect any difference in the reactivity of the subunits, and possibly to establish a dependence of the inactivation on one of them, samples of holo- and apo-glyceraldehyde-phosphate dehydrogenase at different degrees of inactivation were submitted to gel electrophoresis in sodium dodecyl sulfate The stained bands were cut out and, after solubilization of the gel, submitted to scintillation counting The data of Table III show that low levels of inactivation the 37 000 molecular weight subunit is more extensively labelled, while at the end of the inactivation process both subunits are almost equally alkylated Hence it can be concluded that the reaction takes place first at the minor subunit

TABLE III

LABELLING OF THE TWO SUBUNITS OF GLYCERALDEHYDE-PHOSPHATE DEHYDROGENASE BY IODO-[$^{14}C_2$]ACETIC ACID, AS A FUNCTION OF THE DEGREE OF INACTIVATION

For experimental details see Fig 3

	Residual activity (%)	Labelling of subunits (% of total label)	
		M_r 37 000	M_r 43 000
Holoenzyme	80	78	22
	63	67	33
	50	63	37
	25	58	42
	11	52	48
Apoenzyme	85	82	18
	70	72	28
	55	69	31
	22	54	46
	7	53	47

Discussion

The results reported here indicate that the removal of bound pyridine coenzymes from native chloroplast glyceraldehyde-phosphate dehydrogenase causes a conformational change which does not affect the aggregation state of the protein, while it modifies the reactivity of the essential cysteine residues towards iodoacetic acid

Both native and coenzyme-free enzyme are inactivated following a pseudo-first-order process but, the rate of inactivation as well as the affinity for the reagent are lower in apo- than in holoenzyme

Several studies on glycolytic glyceraldehyde-phosphate dehydrogenases [24–29] lead to the proposal that NAD specifically directs iodoacetic acid towards the active site of the enzyme [28] In our case, NADP, which is more effective than NAD in raising the inactivation rate of apoprotein, is also found to enhance the rate of the reaction between native enzyme and iodoacetic acid This may be a strong, although not conclusive, evidence that there is no difference in the coenzyme binding sites and that NAD, which is probably more loosely bound, may be replaced by NADP at any of them As a logical consequence, the conclusion could be drawn that the maximal exposure of the active cysteine residues to the reagent is attained in the holoenzyme species in which NADP occupies all the coenzyme binding sites

On removal of bound pyridine nucleotides not only the inactivation rate, but also the extent of carboxymethylation appears to be modified, as indicated by the finding of four carboxymethylated cysteine residues in apoenzyme maps However, comparison between peptide maps, suggests that apoprotein inactivation is dependent upon the reaction of the same two cysteine residues which become labelled in holoenzyme The additional labelling in the former might be due either to the exposure of cysteine residues which are unavailable for reaction in holoenzyme because they participate in coenzyme binding, or to the fact that pyridine nucleotides enhance the reactivity of the first two residues

Kinetic evidence seems to be in contrast with structural data the former are consistent with alkylation of a single amino acid in the active site, the latter shows that at least two cysteine residues,

located in different tryptic peptides, are labelled in inactive protomer Since this is made up of two subunits differing in molecular weight, the simplest assumption would be that there is one active site cysteine residue in each of them the finding of two labelled spots in peptide maps would suggest a different amino acid sequence around these residues This obviously implies a large difference in the primary sequence of the subunits, as we have suggested on the basis of tryptic peptide maps [6]

On the contrary, some results obtained on enzyme from *Hordeum vulgaris* and *Sinapis alba* lead Cerff and Chamber [8] to the conclusion that the two subunits are structurally identical, the unique difference being the presence of an additional terminal sequence in the major of them if this suggestion is correct, our finding of two different reactive cysteine residues per protomer could be explained by the assumption that alkylation at the active site of both the subunits causes another cysteine to become reactive

Whatever their structural properties are, it is of interest to stress the different reactivity of the two subunits towards iodoacetic acid as suggested by the incorporation of the radioactivity in each of them Since similar results are obtained in native and in apoenzyme, the possibility of an assymetry induced by pyridine coenzyme binding must be excluded

Similarly to glycolytic enzymes [24–33], the affinity of the chloroplast glyceraldehyde-phosphate dehydrogenase for pyridine coenzymes is modified upon carboxymethylation this may be achieved when at least two cysteine residues per 80 000 are reacted, since this is the minimum extent of alkylation found in inactive apoprotein recovered by affinity chromatography On the other hand, the same experiments indicate that incorporation of about 0 5 carboxymethyl residues per protomer neither modifies the activity, nor affects the capacity to bind coenzymes (Table II)

Such findings are difficult to explain unless the relative contribution of the single subunits as well as their assembly within the catalytically-active species of the enzyme are known

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