The Effect of a Paramagnetic Center of 2.03 Average g-Value on d-Glyceraldehyde-3-Phosphate Dehydrogenase

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On the effect of nitrogenoxide and ferrous ion a paramagnetic center of 2.03 average g-value was formed in d-glyceraldehyde-3-phosphate dehydrogenase.

The SH- and histidyl groups of the enzyme took part in the formation of the paramagnetic center as coordination ligands.

The number of paramagnetic centers of the enzyme increased in time because first the most reactive and most readily accessible ligands reacted only. After the modification of the steric structure of the protein paramagnetic complexes were formed also on the sterically hindered ligands.

The formation of paramagnetic center inhibited the activity of the enzyme and this inhibition was competitive with the substrate whereas it was of a mixed type with the coenzyme and phosphate ion.

Introduction

A paramagnetic center of 2.03 average g-value has been observed in the ESR spectrum of yeast cells (Vanin, Nalbandyan, 1965; 1966) and localized in the mitochondrium (Lisovskaya et al., 1970a). This center could be also detected in transplantable tumor cells (Saprin et al., 1968), in the tissue of rodents in the early stage of carcinogenesis (Vithayathil et al., 1965), moreover in normal rabbit liver tissue. The study of the ESR spectra of cell components revealed that under certain conditions a part of the iron and sulphur contained in the so-called "non-heme iron" proteins could be detected in the form of this center (Vanin et al., 1967). The complex formed during carcinogenesis was shown to be bound to extramitochondrial protein fractions containing SH groups (Woolum, Commoner, 1970).

New paramagnetic centers, among others the one of 2.03 average g-value, appeared in various tissues and cells after incubation in nitrogen oxide (Azhipa et al., 1966; Vanin, Chetverikov, 1968; Lisovskaya, Vanin, 1969). This phenomenon could be explained by the fact that NO formed a paramagnetic complex with ferrous ion in the presence of coordination ligands (McDonald et al., 1965). In samples of biological origin the iron bound in non-heme structures (non-heme iron) can only act as ferrous ion in the complex while the sulphur contained in SH-groups and the nitrogen bound in imidazol groups or in free amino-groups can

serve as coordination ligands. Thus the complex is called non-heme iron nitrosyl or iron-nitric oxide or g 2.03 paramagnetic center. As the ESR signal of this center was easily detectable the incubation in nitrogen monoxide was suggested for the detection of non-heme iron proteins (Vanin, 1967). Indeed, this method has been successfully employed to reveal functional differences in the nitrogen fixation of microorganisms and the respiration of yeast cells (Ivleva et al., 1969; Liskovskaya et al., 1970).

Thus the above paramagnetic center is not only of biological interest but it has a methodical significance as well. The study of this paramagnetic center is rather difficult since the biological objects investigated so far (cell suspension, tissue homogenate) were too complex, whereas the chemical models (cysteine-Fe-NO complex) could hardly disclose its biological role. The paramagnetic center was also formed on peptides and isolated proteins on the effect of Fe^{++} and NO (Vanin, 1967). The SH-groups were found to be involved in the center and their signal in the ESR spectrum could clearly be identified. Indirect evidence led to the assumption that another part of the spectrum was the signal of the complex formed on the imidazol groups (Woolum et al., 1968). However, the number and type of SH- or imidazol groups taking part in the formation of the complex are unknown and it is not clarified either how this influences the function of the molecule. Since the knowledge of these facts would not only indicate the limits of applicability of the method in the detection of non-heme iron, but would also answer the question whether this method could be used for the investigation of structure and function, we endeavoured to study the paramagnetic center on a simple biological model, a crystalline enzyme. Glyceradehyde-3-phosphate dehydrogenase was adopted, as this enzyme had been thoroughly investigated, its amino acid sequence, mechanism of action and its probable active center were fairly well known, even the changes in its steric structure on the effect of various blocking agents has been characterized (cf. relevant chapters in the textbooks and reviews: Dévényi et al., 1969; Sund, 1970).

Materials and methods

D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) was prepared from pig muscle and recrystallized four times (Elődi, Szörényi, 1956). Glyceraldehyde-3-phosphate (GAP) was prepared from fructose-1,6-diphosphate (Reanal) as described by Szewczuk et al. (1961). NAD was a product of Reanal, diethyl-pirocarbonate (DEP) was produced by Schuchardt, monoiodoacetic acid by Light, p-chloro-mercuri-benzoate (p-MB) was a product of British Drug Houses, Ltd. All other chemicals were reagent grade commercial preparations.

The paramagnetic complex was prepared according to Woolum et al. (1968). The pH of the ascorbic acid solution needed for the generation of NO was adjusted to $6.0-6.8$ with 5 N NaOH. In ESR experiments the solutions of the enzyme, ascorbic acid, ferrous sulfate and sodium nitrite were mixed and the mixture was

immediately transferred into capillary tubes and then incubated. The reaction was stopped by dipping the capillaries into liquid nitrogen.

The formation of paramagnetic center could not be executed with enzyme dissolved in a buffer solution as the buffers usually employed also formed paramagnetic centers with nitrogen monoxide and ferrous ion. $(NH_4)_2SO_4$ behaved in a similar manner, therefore the experiments were performed with enzyme dissolved in distilled water and gel-filtered on a Sephadex G-50 column equilibrated with distilled water. Since several of the reagents used could also form paramagnetic centers the mixture was also dialyzed or gel-filtered after treatment with iodoacetic acid, p-MB or DEP, and the complex was prepared thereafter.

Above 0 °C the spectra were registered by means of a JES-ME-3X ESR Spectrometer, while the refrigerated samples were analyzed in a JES-P-10 ESR Spectrometer. The ESR signal was registered simultaneously with a $Mn^{++}-$ MgO control, the third line of which is at $g = 2.03$. The amplitudes of the signals of the different samples were normalized to the Mn^{++} line. Thus the measurements could be compared quantitatively. The relative height or amplitude of the signal is the distance between the maximum and minimum of the spectrum as projected to the ordinate. The amplitude of the signal is, strictly speaking, proportional to the concentration of the paramagnetic center only after the double integration of the spectrum. However, as a first approximation, we calculated the relative height of the signal as a quantitative datum and plotted in the figures, since the line-width did not change with pH, temperature or enzyme concentration.

Enzymic activity was measured as described previously (Keleti, Batke, 1965). The SH-groups were determined by titration with p-MB (Boyer, 1954), whereas DEP was employed for the determination of histidines (Ovádi et al., 1967).

For the inhibition experiments GAPD was dissolved in distilled water at pH 6.0. The paramagnetic center was prepared by incubation of 3.75 ml of a 12.0 mg/ml GAPD solution with a mixture of 0.25 ml of a 180 mg/ml ascorbic acid solution pH 6.0, 0.25 ml of a 3.2 mg/ml $FeSO_4$ solution and 0.25 ml of a 180 mg/ml $NaNO_2$ solution at 0 °C (Woolum et al., 1968). After incubating for 10 minutes the mixture was diluted 300 to 400 fold with glycine buffer, pH 8.5, or tris buffer. The activity was assayed with 0.1 ml of diluted mixture in 3 ml final volume in silica cells of 10 mm light path in a Hilger UVISPEK or Opton PMQ II spectrophotometer.

Results

The signals of paramagnetic centers obtained with 10 mg/ml cysteine, 30 mg/ml histidine and 30 mg/ml enzyme, respectively, are shown in Fig. 1. The spectra were registered on refrigerated samples. The half-width of the histidine spectrum (the distance between the two most distant extreme values on the first derivative spectrum, cf. Hedvig, Zentai, 1969 p. 51) is about 57 Gauss indicating a not

completely resolved triplet. The signal of cysteine has a half-width of 40 Gauss only, an unresolved doublet. The spectrum of the enzyme is composed of these two signals, its half-width is somewhat wider, 60 to 65 Gauss, and the signal of cysteine predominates in it. In liquid enzyme samples the width of the spectrum is the same.
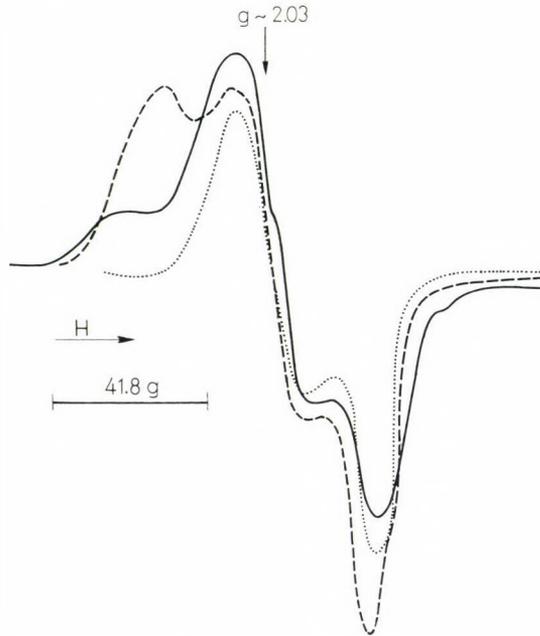

Fig. 1. ESR signal of the $g \approx 2.03$ center obtained with cysteine, histidine and GAPD in refrigerated samples. The paramagnetic complex was prepared as described in Materials and Methods. ———— the signal of 30 mg/ml GAPD in the 2nd hour; - - - the signal of 25 mg/ml histidine; . . . . . . the signal of 10 mg/ml cysteine both in the 5th minute. The arrow under H shows the direction of the increasing magnetic field. The g-values changed in the opposite direction. It is apparent that the signal due to histidine has also a peak at higher g-values, by which it can be distinguished from that of cysteine

Fig. 2 shows the time course of the change in the amplitude of the signal obtained with the amino acids and the enzyme, as well as the decrease of enzyme activity with time. The signal of cysteine decreased slowly whereas that of histidine very rapidly. The signal of the enzyme rapidly increased for thirty minutes, then the increase slowed down and the signal reached a plateau value after two hours. The amplitude of the signal after two hours was about tenfold the value measured after five minutes. The center could be maintained for a prolonged time only in a closed capillary tube because of its sensitivity to oxygen. The complete signal-form of the enzyme samples developed in 5 to 10 minutes; the peak characteristic of histidine and observable at lower field strength ($g \approx 2.04$) appeared beside the signal
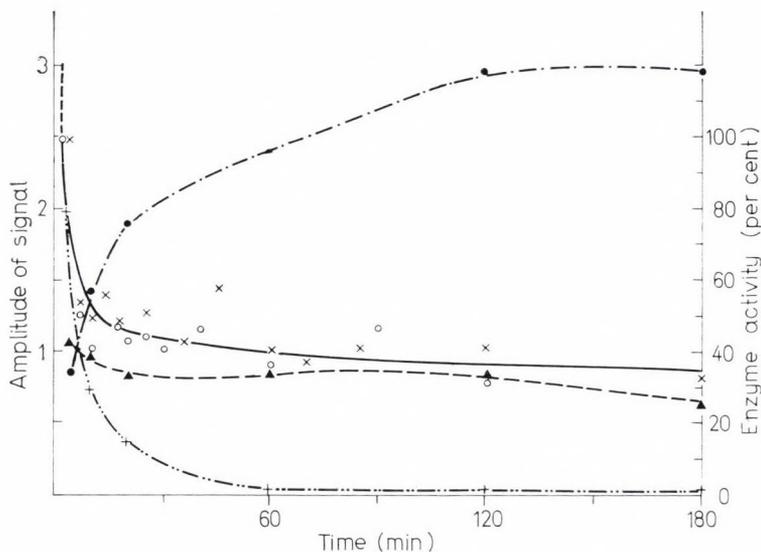
Fig. 2. Time course of the change in the amplitude of the signal of the $g \approx 2.03$ paramagnetic complex. The amplitude of the signal of 30 mg/ml GAPD, ● ·— ·●; 25 mg/ml histidine, +··—··+; and 10 mg/ml cysteine ▲ — ▲. The enzyme activity in per cent is shown on the ordinate on the right side. After mixing the components as described in Materials and Methods samples were taken from the mixture at various times and the activity of the samples was measured after a 300-fold dilution in glycine buffer. The first point was measured 2 to 3 minutes after the preparation of the mixture. The activity of this sample was taken as 100 %. The two different symbols (×, o) refer to enzyme activities measured in two separate experiments

of cysteine after 5 to 10 minutes only. On further incubation the form of the signal did not change, which pointed to the fact that only the number of paramagnetic complexes, not their character, had changed.

More than 70 per cent of the enzymic activity is lost on the effect of the paramagnetic center and this inhibition developed in about 10 minutes. The inhibition attained its maximum between 7 and 10 minutes. The activity only changed to a slight extent during the following one to three hours.

These data led us to the conclusion that first the SH-groups of the enzyme participated in the formation of the paramagnetic radical followed by the reaction of the histidyl groups. These reactions were responsible for the inhibition of enzymic activity. Further on the steric structure of the enzyme changed as a secondary process, as a result of which, paramagnetic centers were formed on more SH- and histidyl groups, but the enzyme activity did not further decrease.

The development of the signal of the enzyme at various pH values is shown in Fig. 3. In alkaline medium the ESR signal was smaller and it was characteristic of cysteine. The complex was less paramagnetic than in the previous cases. The ESR spectra remained in the protein fraction after gel-filtration of the sam-

ples on a Sephadex-column, which proved that the center was formed on the enzyme.

Fig. 4 shows the signal height of the complex formed on the enzyme at various temperatures. The amplitude of the signal of the enzyme denatured by heat at 60 °C did not vary in time, whether the enzyme was incubated at 60 °C or it was put into a water bath at 60 °C for 3 minutes after incubation at 0 °C. The amplitude of the signal was greater at the 5th minute in the heat-denatured sam-
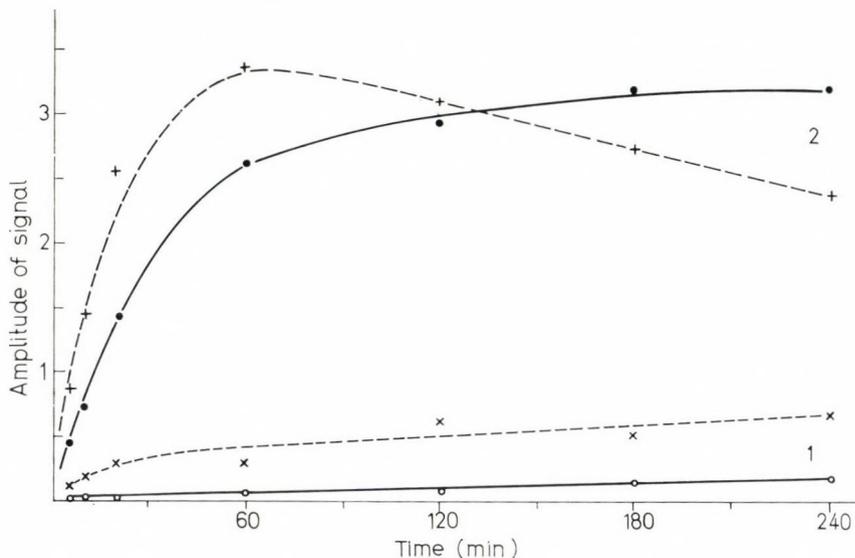

Fig. 3. Time course of the change in the amplitude of the signal of the complex of $g \approx 2.03$ at various pH values. The pH of the mixture was adjusted with 5 N NaOH or 0.1 N TRIS or 0.1 N HCl solutions
1) Alkaline range $\times$ − − $\times$ pH 8; o ———— o pH 9
2) Acide range + − − + pH 6; ● ———— ● pH 6.8

ples than in the native enzyme, but later on it did not increase and remained characteristic of cysteine as in the first minutes. The amplitude of the signal of samples incubated at 0 °C and then denatured by heat decreased as compared to that of the native and the peak characteristic of histidine disappeared.

Since denaturation was carried out at a temperature different from that at which the native enzyme was studied the possibility had to be excluded that the decrease in the amplitude of the spectrum could be due to the difference in temperature. The temperature dependence of the signal of the heat-denatured enzyme is shown in Fig. 5 in the temperature range −140° to + 98 °C. The variation in the amplitude of the signal of the complex followed Curie's law, but the temperature dependence was negligible between 0 ° and 60 °C.

Fig. 6 shows the time dependence of the signal of the enzyme treated with 10 mole equivalents of monoiodoacetic acid, 4 mole equivalents of $AgNO_3$
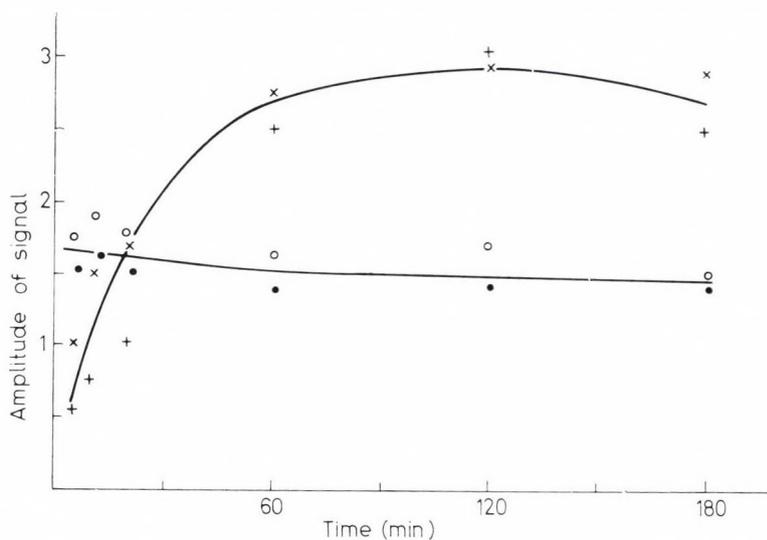
Fig. 4. The effect of heat denaturation on the amplitude of the $g \approx 2.03$ signal of GAPD. $+$, $\times$ samples incubated at 0 °C (control); ● — ● samples incubated at 60 °C; o — o enzyme incubated at 0 °C. The latter preparation was immersed in a water bath at 60 °C for 3 minutes before refrigeration. The duration of incubation is indicated on the abscissa. The preparation and measurement of the complex were performed as described in Materials and Methods, i.e. the spectra were registered on refrigerated samples independently of the temperature of incubation
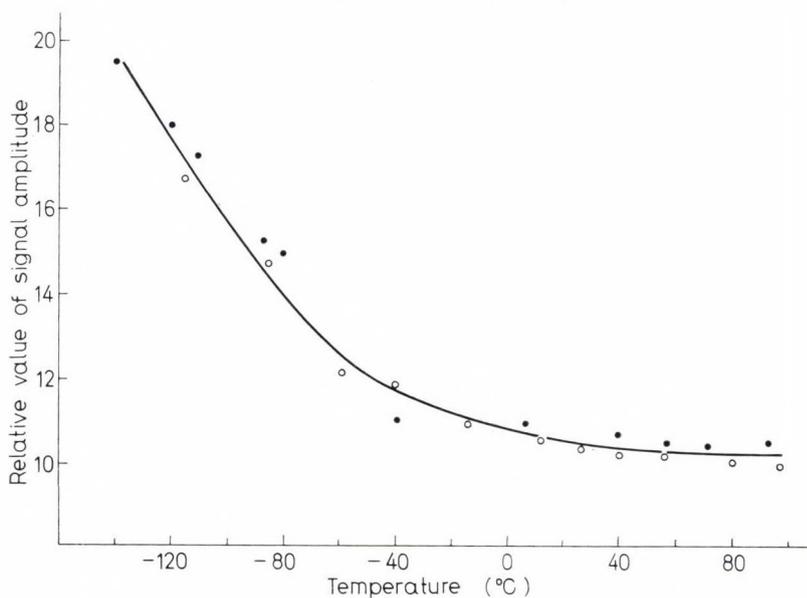

Fig. 5. Relative values of the amplitude of the signal obtained with denatured enzyme as a function of temperature. The complex was denatured in a water bath at 60 °C. The spectrum of the complex was measured at the temperatures indicated on the abscissa
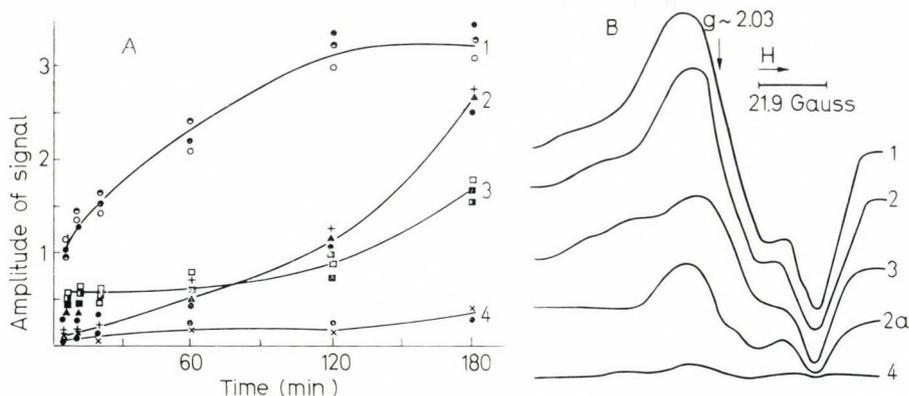
Fig. 6. Time course of the amplitude of the signal of the enzyme complex incubated with various blocking agents and without treatment, respectively. After the blocking carried out as described in Methods the pH of the enzyme solution was adjusted to 6, and then the complex was prepared. A) The various symbols represent independent experiments. 1. Native enzyme; 2. Carbethoxylated enzyme, 3. Enzyme treated with monoiodoacetic acid or silver ions; 4. Mercaptidated enzyme. B) ESR spectra measured in refrigerated state. 1. Native enzyme after incubation for 180 min; 2. Carbethoxylated enzyme after 180 min of incubation and 2 a refrigerated at the 120th minute of the incubation; 3. Carboxymethylated enzyme refrigerated at the 180th minute; 4. Mercaptidated enzyme after 180 min of incubation

100 mole equivalents of DEP and 20 mole equivalents of p-MB, respectively. All these blocking agents hindered the development of the signal of the complex as compared to the native enzyme. The mercaptidation (p-MB-treatment) caused a permanent inhibition of the development of the ESR signal. If the development of the complex was started when the mercaptidated enzyme had already become opalescent or it had precipitated, the signal characteristic of histidine was observed in the spectrum. The carbethoxylation (DEP-treatment) of the enzyme inhibited the appearance of spectra characteristic of histidine and cysteine only temporarily. The enzyme treated with DEP exhibited a small cysteine-type signal which gradually increased, and then around the 180th minute the histidine-type signal suddenly appeared. Carboxymethylation (monoiodoacetic acid-treatment) and Ag ion decreased only the signal characteristic of cysteine. In this instance the amplitude of the histidine signal was equal to that obtained with the native enzyme, while the amplitude of the signal characteristic of cysteine was only one half of that. On further incubation the intensity of the spectra decreased (not shown in the figure). Some characteristic spectra are shown in the insert of the figure.

Among the reagents required for the formation of the paramagnetic center, $FeSO_4$ (in tris buffer in the presence of arsenate) strongly innhibited the activity of the enzyme. Under similar circumstances, or in the presence of phosphate ion, ascorbic acid had the same effect. $NaNO_2$ alone did not inhibit the activity of the

enzyme in any of the cases studied. Likewise, inhibition could not be detected either with $FeSO_4$ or with ascorbic acid when these reagents were applied separately and the enzyme activity was measured in glycine buffer in the presence of
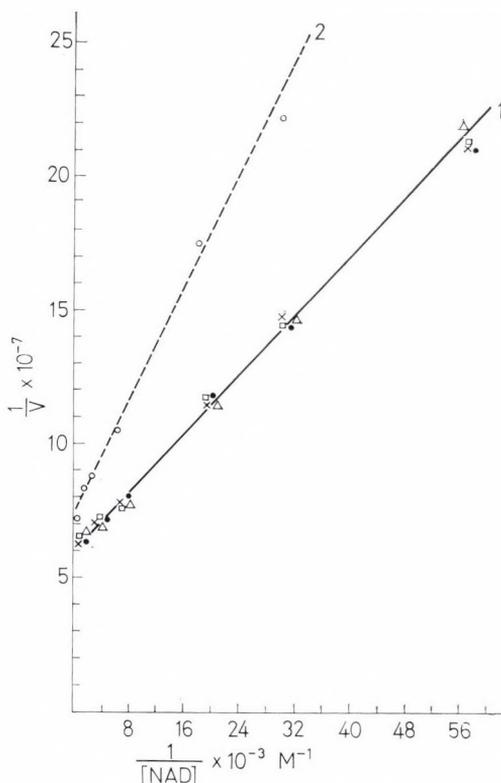

Fig. 7. Inhibitory effect of the paramagnetic center with respect to NAD as plotted according to Lineweaver and Burk. The paramagnetic center was prepared as described in Methods. The mixture was diluted with 0.1 M glycine buffer, pH 8.5, after incubation for 10 minutes. The measurement of activity was performed at a final GAPD concentration of 1 to 3 μg/ml. The results are normalized to the same protein concentration. GAP: 1.38×10^{-3} M; phosphate ion: 1×10^{-2} M. v = moles of NADH formed per ml in 15 sec. 1. $\times$: native GAPD; $\square$: GAPD + $FeSO_4$; $\bullet$: GAPD + ascorbic acid; $\triangle$: GAPD + $NaNO_2$. 2. o GAPD containing paramagnetic center

phosphate ion (Fig. 7). Therefore we investigated the effect of the paramagnetic center in glycine buffer and in the presence of phosphate.

The inhibition of enzyme activity caused by the paramagnetic center was of a mixed type with both NAD (Fig. 7) and phosphate ion (Fig. 8), whereas the inhibition was competitive with the substrate (Fig. 9).
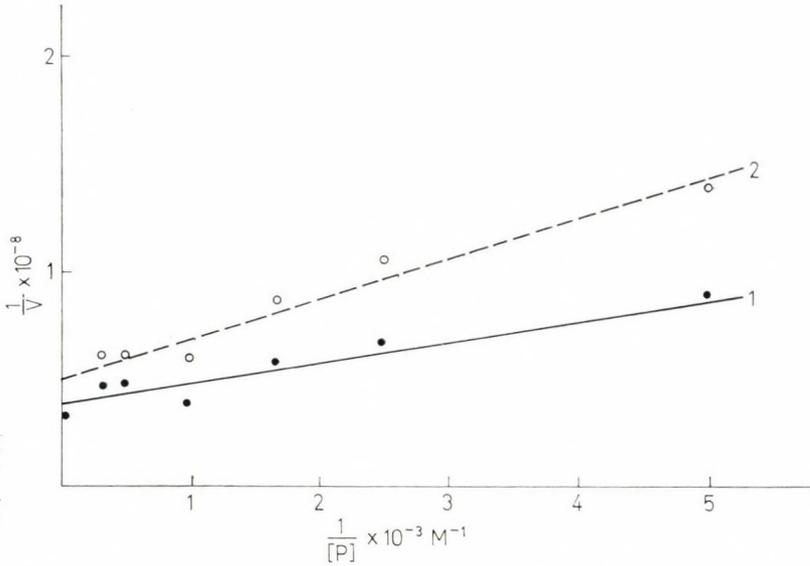
Fig. 8. Inhibitory effect of the paramagnetic center with respect to phosphate ion as plotted according to Lineweaver and Burk. The experimental conditions and the symbols are the same as in Fig. 7. NAD: 1.48×10^{-3} M; GAP: 1.38×10^{-3} M
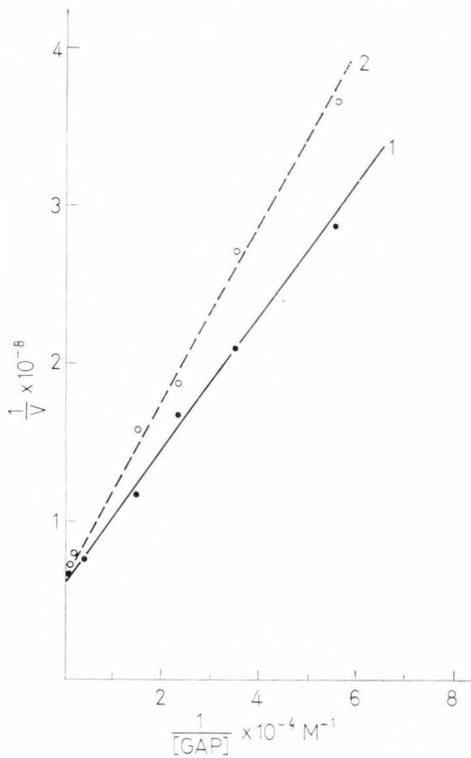

Fig. 9. Inhibitory effect of the paramagnetic center with respect to GAP as plotted according to Lineweaver and Burk. The experimental conditions and the symbols are the same as in Fig. 7. NAD: 1.48×10^{-3} M; phosphate ion: 1×10^{-2} M

Since the oxidized substrate forms an acylenzyme with the SH-group of GAPD and the paramagnetic center exhibits competitive inhibition with the substrate, we examined whether the number of SH-groups titratable with p-MB changed on the effect of the paramagnetic center. Native GAPD contains 16 SH-groups per molecule. Ten minutes after the formation of the paramagnetic center approximately half of the SH-groups disappeared (Fig. 10). The titration performed in the second hour also showed that 8 out of the 16 SH-groups were blocked.
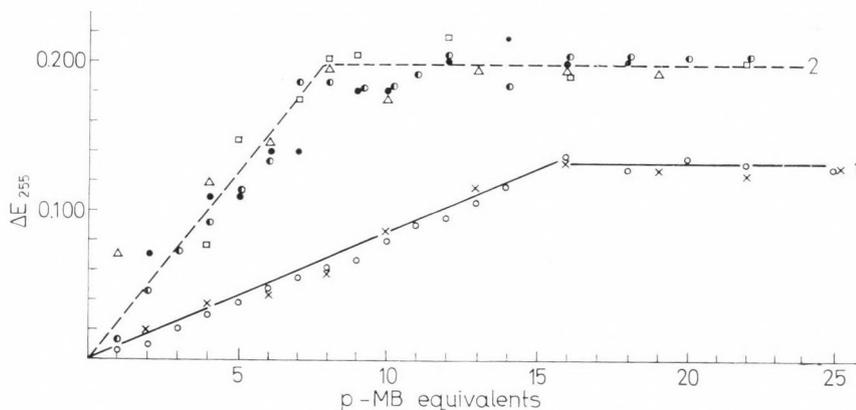

Fig. 10. Determination of the free SH-groups of GAPD. A solution containing about 300 μg/ml protein was incubated with various amounts of p-MB as indicated in the Figure, in 0.1 M glycine buffer, pH 8.5, at 0 °C for 1 hour. 1: native GAPD; 2: GAPD containing paramagnetic center. Since the absorbance of the paramagnetic center was great at 255 nm the differences in absorption were read partly after dilution with buffer and partly after gel-filtration on a 15×1.5 cm Sephadex G-50 column. The different symbols indicate independent experiments normalized to the same protein concentration

Since histidine is carbethoxylated in the reaction with DEP, i.e. a covalent bond is formed, it was probable that DEP abolished the paramagnetic center even if it was bound to histidine. Indeed, as a maximum 23 to 24 histidine residues could be detected with DEP on the surface of both the native enzyme and that containing the paramagnetic center.

Discussion

The study of the spectra of the complexes formed with the enzyme and with the two amino acids, respectively, confirmed the results of Vanin (1967) and Woolum et al. (1968) obtained with peptides. The enzyme GAPD studied by us is composed of four subunits; each of them contains 4 SH and 11 imidazol groups (Harris, Perham, 1968). Thus both ligands have to be considered as participants in the formation of the paramagnetic center. If both SH and imidazol groups are present, the signal of the former is predominant in the ESR spectrum.

The ESR signal of the amino acid complex was narrower in liquid medium than in the refrigerated state and showed a superfine structure. The enzyme complex did not show a superfine structure either in the liquid or in the frozen state and the width of the signal was the same in the two cases. The phenomenon indicates that the complex was formed on the protein molecule since the motion of the centers bound to the enzyme got "frozen". Therefore the signal was also broadened in liquid medium and the anisotropy of the g-tensor could be seen from the spectra (cf. Hedvig, Zentai, 1969). The appearance of an unresolved doublet in the signal of the cysteine complex led to the conclusion that the center was axially symmetrical, whereas the triplet signal of the histidine comples pointed to the existence of different g-values in all the three main directions (Chetverikov et al., 1969; Kneubühl, 1960; Woolum et al., 1968).

The signal obtained with compounds of small molecular weight (urea, guanidine-HCl, etc.) and with amino acids decreased in time, while the signal of the protein complex was more stable. The disappearance of the paramagnetic centers is assumed to be caused by recombination as a diamagnetic center is formed from two paramagnetic ones (McDonald et al., 1965). This process is inhibited in the case of centers bound to macromolecules, since the collision of such centers is rare. This phenomenon accounts for the fact that the histidine complex formed on the protein molecule is also stable. However, the gradual increase of the signal cannot be explained by the stability of the centers. According to our knowledge, there are no data in the literature about such an increase of the signal. One of the four SH-groups of a subunit of GAPD is especially reactive and two of them are located on the surface of the molecule, whereas the other two SH-groups are located inside the molecule (Racker, 1954; Friedrich, Szabolcsi, 1967; Boross et al., 1969). In the case of native GAPD only 6 out of the 11 histidyl groups of a subunit are found to be reactive (Ovádi et al., 1967; Ovádi, Keleti, 1969). Thus the groups which may participate in the formation of the paramagnetic center are partly buried inside the molecule. We assume that the increase of signals is brought about by the unmasking of buried groups due to the gradual unfolding of the enzyme molecule during the complex formation.

These were two ways to prove this hypothesis. The first possibility was to destroy the structure of the protein by denaturation and then to observe the formation of the complex by means of the ESR signal. We tried a number of denaturing agents but they could not be used if the complex was prepared according to Woolum et al. (1968), since paramagnetic complexes were also formed on the denaturing agents (guanidine hydrochloride, urea, dimethylformamide, dioxane) and thus the evaluation of the spectra was rendered impossible. In these solutions an intensive signal could be detected even in the absence of the proteins, rapidly decayed in time. In the presence of protein the ESR signal also disappeared rapidly and the complex was transformed into a diamagnetic form. Other denaturing agents (sodium dodecyl sulfate) could prevent the formation of the paramagnetic complex. Denaturation by heat was the only method that could be applied. In the first minutes the signal of the GAPD samples denatured by heat was indeed

greater than that of native GAPD, however, it did not change in time. On the other hand, the signal of the complex formed on the native protein decreased after heat treatment. This phenomenon was also observed by Vanin (1967) on lyophilized samples. Since the amplitude of the signal changes only slightly from 0 to 60 °C according to Curie's law, the decrease in the number of paramagnetic complexes was caused by a sudden conformational change of the protein. The complete disappearance of the histidine peak showed that this paramagnetic center was also labile on the protein as it completely disappeared on denaturation or lost its paramagnetic character.

The second possibility for the investigation of the role of the masked groups in the formation of the complex was to block the SH-groups with monoiodoacetic acid or silver ions or p-MB, while the imidazol groups with diethyl pyrocarbonate (DEP). With monoiodoacetic acid or 4 equivalents of Ag^+ only the four reactive SH-groups of the native GAPD molecule could be blocked (Racker, 1954; Boross, 1965), whereas p-MB could block all the SH-groups (Boyer, Segal, 1954). In fact, the treatment of the enzyme with monoiodoacetic acid or silver ion did not prevent the appearance of the cysteine signal, only decreased it by fifty per cent. The treatment with monoiodoacetic acid or with silver ions did not change the steric structure of the enzyme. In these cases four free, non-reactive SH-groups are on the surface of the enzyme beside the four blocked reactive ones.

Hence we interpret the fact that the cysteine-type signal slowly developed after the above treatment and attained only one half of the value obtained in the case of the native enzyme by assuming that the paramagnetic centers were formed only on the four accessible but non-reactive SH-groups. Consequently, the four reactive SH-groups of the native enzyme form complexes first, whereas the reaction of the other four free SH-groups is slow. The change in time of the amplitude of the signal is thus caused by the fact that the formation of the complex is much more rapid on the reactive SH-groups than on the non-reactive ones.

After treatment with 20 mole equivalents of p-MB no signal, or only that of the histidine complex could be detected. The mercaptidation of all SH-groups of the enzyme causes profound changes in the steric structure. In this case no more SH-groups can be unmasked. It seems probable that p-MB sterically hinders the histidine groups in the non-precipitated enzyme and they become accessible only after precipitation.

The blocking with DEP mainly inhibits the development of the signal in the beginning, but later the whole spectrum characteristic of the enzyme can be detected, though the amplitude of the signals is smaller. DEP can only block the imidazol groups located on the surface of the enzyme, but some of these are near the reactive SH-group hindering its accessibility (Ovádi, Keleti, 1969).

However, carbethoxylation changes the steric structure of the enzyme which is proved by the fact that both the SH-groups and some of the previously hindered histidine groups could react during incubation and formed paramagnetic centers.

The number of the SH-groups decreases during the formation of the com-

plex as detected by titration with p-MB. However, we failed to demonstrate the same for histidyl groups during this process by means of titration with DEP. DEP forms a covalent bond with histidine and thus the complexes are probably destroyed by the reagent. The fact that 8 out of the 16 SH-groups disappeared during the formation of the complex is in agreement with the results of carboxymethylation and of the treatment with silver ions. In both modification reactions only the free SH-groups of the enzyme seem to react. It should be noted that the number of SH-groups titrated after two hours does not provide full evidence. After such prolonged incubation the protein was precipitated during gel-filtration and the titration with p-MB could be performed in 8 M urea only. We could not decide whether more SH-groups did not react during the prolonged incubation or the paramagnetic center was removed from the SH-groups reacting later on the effect of denaturation, or on the effect of urea.

The mere finding that only the free SH-groups reacted does not exclude that the formation of the paramagnetic center causes a change in the steric or quaternary structure of the enzyme. The complete extinction of the excimer fluorescence of the enzyme on the effect of the paramagnetic center (Keleti, 1970) points to this phenomenon.

The results of the enzymological investigations are in agreement with the above statements. The formation of the paramagnetic center causes a competitive inhibition with the substrate only, as the substrate reacts during its oxidation, with the reactive SH-group. A mixed type inhibition was found with the coenzyme and with the phosphate ion, i.e. a competitive component was also involved. This phenomenon can be explained on the one hand by the fact that both substances are bound to the active center containing the reactive SH-group, thus steric hindrance may also occur. On the other hand the paramagnetic center can also be formed on histidine which could participate in the binding of phosphate ion. The fact that total inhibition of the enzyme not reached even after a long incubation can be probably explained by assuming that the paramagnetic centers were partly removed from the enzyme by the competitive GAP and by NAD and phosphate exhibiting a competitive component in experiments made in the presence of optimal substrate concentrations. However, it is also possible that the glycine buffer employed in the measurement of enzyme activity removed paramagnetic centers from the enzyme.

At any rate, these observations do not exclude the hypothesis of Woolum and Commoner (1970) according to which the center may have a regulative role in the activity of certain enzymes even under physiological conditions.

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