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INVESTIGATION OF THE CONFORMATION OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

I. A. BOLOTINA, D. S. MARKOVICH, M. V. VOLKENSTEIN AND P. ZAVODSKY
Institute of High Molecular Compounds, Academy of Science of the U.S.S.R., Leningrad (U.S.S.R.)

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SUMMARY

The conformation of D-glyceraldehyde-3-phosphate dehydrogenase was studied by means of spectropolarimetry under various conditions. It was shown that at neutral pH the enzyme possessed a compact globular structure with approx. 40% α-helical content. When the pH was altered, a change in secondary and tertiary structure was observed which was manifested in the change of α-helicity and in the uncoiling of the molecules. Sodium dodecyl sulphate produced a change in the tertiary and quaternary structure, *i.e.* uncoiling of the molecules and the division of the enzyme molecule into subunits, while the secondary structure remained unchanged. The coenzyme (NAD^+) and the substrate (glyceraldehyde) produced conformational changes in the apoenzyme molecule. The results can be interpreted in terms of KOSHLAND's "induced-fit" hypothesis.

INTRODUCTION

The modern concept of the nature of enzymic activity is based on the assumption of conformational fluctuations in the enzyme macromolecules. According to this hypothesis, substrate, coenzyme and other effectors stabilize certain conformations, the structural fitness between the enzyme and the effector being obtained (according to KOSHLAND[1-3]). KOSHLAND's hypothesis has had several indirect confirmations[2,4,5]; there are, however, few direct proofs of a change in the structure of the enzyme caused by its interactions with the effectors[6-8].

The aim of this work was to study the structural changes in GAPD (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) caused by changes in pH and by the influence of coenzymes and substrate. We used the spectropolarimetric method as giving direct information concerning molecular structure.

GAPD catalyzes the reaction of oxidation of D-phosphoglyceraldehyde proceeding in two stages:

Abbreviations: GAPD, D-glyceraldehyde-3-phosphate dehydrogenase; ORD, optical rotatory dispersion.

$$\begin{array}{l} R\text{–}\underset{\underset{O}{\|}}{C}\text{–}H + E\text{–}SH + NAD^+ \rightleftarrows R\text{–}\underset{\underset{O}{\|}}{C}\text{–}S\text{–}E\text{–}NADH + H^+ \end{array} \quad (1)$$

$$\begin{array}{l} R\text{–}\underset{\underset{O}{\|}}{C}\text{–}S\text{–}E\text{–}NADH + HPO_4^{2-} \rightleftarrows R\text{–}\underset{\underset{O}{\|}}{C}\text{–}O\text{–}PO_3^{2-} + E\text{–}SH + NADH \end{array} \quad (2)$$

where R–CHO is the substrate with the aldehyde group, *E* is the enzyme, NAD^+ is the coenzyme (nicotinamide–adenine dinucleotide), and NADH is reduced nicotinamide–adenine dinucleotide.

In the first stage, the coenzyme is bound to the enzyme first, and then the substrate is bound[9], the ternary complex being fairly stable. The oxidation of substrate and the reduction of NAD^+ take place at this stage. The phosphorolysis of the enzyme–substrate bond and the removal of the phosphorylated product (1,3-phosphoglyceraldehyde) proceed in the second stage. This stage requires the participation of orthophosphate or arsenate. The reaction rate is known to be a function of pH. Thus, in this reaction the conformation of the apoenzyme may be tentatively assumed to be affected by the following influences: (1) the influence of NAD^+ upon the apoenzyme; (2) the transformation of NAD^+ into NADH and the action of NADH upon the apoenzyme; (3) the formation of the complex R–CO–S*E* with the substrate and its slow hydrolysis. The present paper deals with attempts to evaluate the parts played by these factors in the conformational changes in the enzyme.

MATERIALS AND METHODS

Materials. GAPD, recrystallized four times, was isolated from pig skeletal muscle according to the method of ELÖDI AND SZÖRENYI[10] at the Biochemical Institute of the Hungarian Academy of Sciences in Budapest. The molecular enzymic activity of GAPD (mol.wt. 140 000) with D-phosphoglyceraldehyde as substrate was 11 000–12 000 at pH 8.5. The calcium salt of D-phosphoglyceraldehyde was obtained from the Biochemical Institute of the Hungarian Academy of Sciences. The preparations of NAD^+ and NADH were obtained from the Lawson Co. (Great Britain) and DL-glyceraldehyde was obtained from Reanal (Hungary). Investigations were conducted in a 0.1 M glycine buffer at various pH values. Native enzyme was crystallized with bound NAD^+ in the molar ratio of 2.5:1. Apoenzyme was prepared by treating 1–2% solutions of native enzyme with activated charcoal according to VELICK AND FURFINE[11]. Commercial preparations of activated charcoal were thoroughly purified[12]. The reconstruction of the GAPD holoenzyme was conducted by adding NAD^+ to the apoenzyme in the molar ratio of 3–5:1. NADH was added to GAPD in the molar ratio of 20:1. DL-Glyceraldehyde used as substrate was added to GAPD (with 2.5 moles of bound NAD^+ per mole) in the ratio 1000 moles of glyceraldehyde per 1 mole of GAPD. $(NH_4)_2SO_4$ was removed from the initial enzyme solution by gel filtration of the solution on columns (2 cm × 20 cm) of Sephadex G-25 saturated with the buffer used. The same columns were used for obtaining enzyme solutions having the desired pH values. This method is milder than direct titration of solutions with alkali or acid. GAPD was denatured by incubation in 8 M urea for 1 h at 30°. Sodium dodecyl sulphate was added to the 1–2% GAPD solutions in the ratio 1/3:1, 1/2:1, 1:1 by weight of the enzyme.

Enzyme concentration was determined with an SF-4A spectrophotometer by

protein extinction in a solution of 0.1 M NaOH at 280 mμ using the value $E^{1\%}_{1\,cm} = 10.0$ (ref. 11).

Enzymic activity was determined with an SF-4A spectrophotometer by WARBURG's optical test[13] from the change in absorbance of the solution at 340 mμ during the first minute from the moment when 1 μg enzyme was added to the solution.

Measurements of ORD were performed with a Pepol-60-type spectropolarimeter (Bellingham and Stanley, Great Britain). A xenon lamp was used as a light source. The rotation angle was determined to within $\pm 0.002°$. The angle of the prism oscillation was 30°. Measurements were conducted in the spectral region from 300 to 590 mμ at room temperature using cells of various lengths (0.5 dm, 1 dm, 2.2 dm) and with quartz end-plates. The enzymic activity of GAPD was determined both before and after measurements. The constants λ_c and K_c in Drude's equation were calculated from the curves of ORD, *viz.*

$$[\alpha]_\lambda = \frac{K_c}{\lambda^2 - \lambda_c^2} \tag{3}$$

as well as the constants b_0 and a_0 in the Moffitt equation, *viz.*

$$[m']_\lambda = \frac{3}{n^2 + 2} \frac{M_0}{100} [\alpha]_\lambda = a_0 \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + b_0 \left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right)^2 \tag{4}$$

and the constants A_{193} and A_{225} in the modified equation of SCHECHTER AND BLOUT[14-16]

$$[m']_\lambda = \frac{A_{193} \lambda_{193}^2}{\lambda^2 - \lambda_{193}^2} + \frac{A_{225} \lambda_{225}^2}{\lambda^2 - \lambda_{225}^2} \tag{5}$$

In some cases the constants A_{193} and A_{225} were estimated from λ_c and K_c by the transition formulae[16]:

$$\begin{aligned} A_{193} &= 0.201 \cdot 10^{-8} K_c (\lambda_{225}^2 - \lambda_c^2) \\ A_{225} &= -0.148 \cdot 10^{-8} K_c (\lambda_{193}^2 - \lambda_c^2) \end{aligned} \tag{6}$$

Circular dichroism was measured with a Jouan-Roussel dichrograph (France) in Professor TUMERMAN's laboratory at the Institute of Molecular Biology (Moscow). 1–1.5% solutions of GAPD were investigated.

Measurements of the sedimentation rate were made with a MOM ultracentrifuge, type OU-101 with a Philpot–Svensson optical system at 20°. Sedimentation coefficient (s) was calculated from the slope of the curve $\ln x = s^2 t$ where x is the distance to the maximum ordinate of the gradient curve, and t is the time. Solutions of various concentrations were investigated and the s values were extrapolated to zero concentration.

Measurements of the diffusion rate were performed with a polarizing diffusometer as described by TSVETKOV, ESKIN AND FRENKEL[17] with interference optics at the GAPD concentration of 0.05% (in a 0.1 M glycine buffer, pH 8.5). The diffusion coefficient (D) was calculated by the method of the maximum ordinate and the zero moment (of the area) of the experimental curves. The molecular weight of GAPD was calculated from the Svedberg formula

$$M = \frac{RTs}{(1 - \bar{v}\varrho)D} \tag{7}$$

where $\bar{\nu}$ is the partial specific colume, R is the gas constant, T is the Kelvin temperature, and ϱ is the density of the solvent.

Measurements of the pH of the solutions were carried out with a pH meter, type LPU-01, to within ± 0.05 unit of pH.

RESULTS

Curves of rotatory dispersion for native GAPD (with bound NAD^+) were obtained for a broad pH interval (from pH 4.0 to pH 10.8) for the apoenzyme of GAPD at pH 8.5, for reconstructed complexes of GAPD–NAD^+ and GAPD–NADH and for the ternary complex GAPD–NADH–D-glyceraldehyde at pH 8.5. These curves were used for calculating the constants λ_c, K_c, a_0, b_0, A_{193} and A_{225}. The values obtained are shown in Table I. All the dispersion curves are well characterized by the

TABLE I

EFFECT OF pH ON ORD PARAMETERS OF GAPD (WITH NAD^+ IN THE MOLAR RATIO 2.5:1)

pH	λ_c	$-K_c \times 10^{-5}$	a_0	b_0	A_{193}	A_{225}
4.0	236	224	−468	− 83	221	−611
5.0	263	127	−244	−193	473	−599
6.0	265	100	−202	−203	398	−493
7.0	268	94	−175	−210	401	−481
7.7	267	93	−180	−200	386	−469
8.7	266	98	−188	−203	396	−485
9.15	267	98	−175	−217	402	−488
9.35	264	113	−177	−193	433	−543
10.0	252	136	−272	−173	355	−533
10.85	230	222	−447	−100	101	−514

simple Drude equation. As Table I and Fig. 1 show, the structure of native GAPD depended on the pH of the medium. In the region of neutral pH (from 6 to 9) the GAPD structure was characterized by comparatively high values of λ_c and $-b_0$ corresponding approximately to 35–40% α-helix content. When the pH was changed to higher or lower values, a change in the secondary and tertiary GAPD structures was observed: λ_c and b_0 decreased, K_c and a_0 increased. The curves of λ_c ($-b_0$) and $K_c(a_0)$ as functions of pH are bell-shaped. It is noteworthy that a change in K_c and a_0 began before the change in λ_c and b_0 (Fig. 1).

Fig. 2 shows data obtained by analysis of the ORD by the SCHECHTER–BLOUT method[14,15]. According to these authors, if the polypeptide chain of the protein is only in the α-helical and coil-shaped conformations, the constants A_{193} and A_{225} in Eqn. 5 should be connected by linear relationships:

$$\text{I. } A_{225} = -0.55 \cdot A_{193} - 430 \text{ (aqueous solutions, } \varepsilon > 30) \quad (8)$$

$$\text{II. } A_{225} = -0.55 \cdot A_{193} - 280 \text{ (organic solvents, } \varepsilon < 30) \quad (9)$$

Fig. 2 shows the straight lines I ($\varepsilon > 30$) and II ($\varepsilon < 30$) of SCHECHTER AND BLOUT and the points expressing the dependence of A_{193} on A_{225} for GAPD at various pH values. As Fig. 2 shows, the points corresponding to pH 6–9 shift from line I and fall on line II.

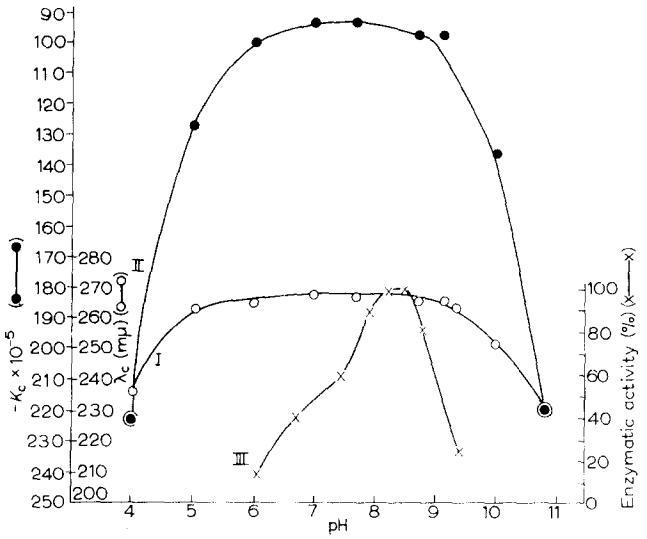

Fig. 1. Curve I (○—○). Dependence of λ_c on pH for native GAPD (with NAD^+ in the molar ratio 2.5:1) in a 0.1 M glycine buffer. Curve II (●—●). Dependence of K_c on pH for native GAPD under the same conditions. Curve III (×—×). Dependence of enzymic activity on pH for GAPD in the oxidation reaction of D-phosphoglyceraldehyde. 100% activity is taken as that giving $\Delta A_{340\ m\mu}$/min = 0.520 for the system: 7 mM D-phosphoglyceraldehyde; 1.66 mM NAD^+; 0.007 μM GAPD; 0.1 M glycine buffer.

Similar results have been recently obtained for glutamate dehydrogenase[18] and for apomyoglobin[19]; we have, moreover, observed the same for lactate dehydrogenase[20].

At pH 10 and pH 5 the points shifted towards line I and at extreme pH values (10.85 and 4) they fell on line I.

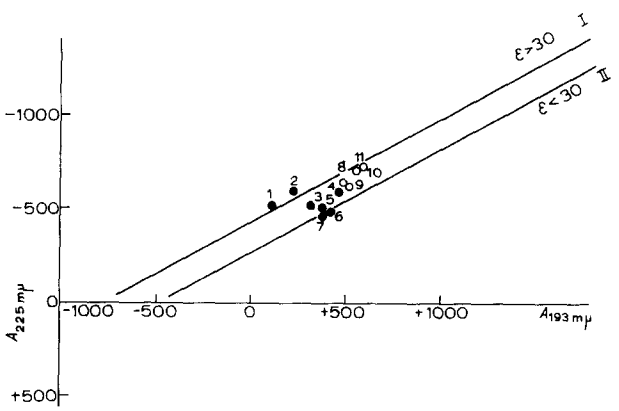

Fig. 2. Plot of SCHECHTER AND BLOUT constants A_{225} *versus* A_{193}. I: Empirical dependence of SCHECHTER AND BLOUT constants $A_{225} = -0.55 \cdot A_{193} - 430$ for solvents with $\varepsilon > 30$. II: Empirical dependence of SCHECHTER AND BLOUT constants $A_{225} = -0.55 \cdot A_{193} - 280$ for solvents with $\varepsilon < 30$. Points 1–7 (●): native GAPD (with NAD^+ in the molar ratio 2.5:1) in a 0.1 M glycine buffer: 1, pH 10.85; 2, pH 4.0; 3, pH 10.0; 4, pH 5.0; 5, pH 9.15, 8.7, or 6.0; 6, pH 7.0; 7, pH 7.7. Points 8–11 (○): native GAPD (with NAD^+ in the molar ratio 2.5:1) in a 0.1 M glycine buffer, pH 8.5, with sodium dodecyl sulphate in the ratio (by weight): 8, 1/3:1; 9, 1/2:1; 10, 2/3:1; 11, 1:1.

This displacement of the points from line I at pH 6–9 may have been due either to the presence of some other ordered structures not belonging to the α-helix type (*e.g.* to the β-form, *cf.* SCHECHTER AND BLOUT[14]) or to the influence of internal local electrical fields of the molecule on the ORD constants. GAPD is known to contain a considerable proportion of aromatic amino acids, creating a strong hydrophobic "nucleus" in the molecule with local ε values characteristic of organic solvents[11]. The presence of such a dense hydrophobic nucleus in the GAPD molecule is corroborated by data on the kinetics of deuterium exchange[21], spectrophotometric titration[22] and iodination of the tyrosine residue[22].

In order to determine the causes of shifting of the points from line I in the SCHECHTER–BLOUT plot, we have conducted experiments with sodium dodecyl sulphate. This is known to influence hydrophobic interactions. It could be expected that sodium dodecyl sulphate would destroy the hydrophobic nucleus of the GAPD molecule, cause the uncoiling of the molecule and thus decrease the influence of the local field upon the ORD constants.

As Fig. 2 shows, when sodium dodecyl sulphate was introduced into the GAPD solution, even in the ratio 1/3:1 by weight of GAPD, a shift of the point towards line I was observed. When the amount of sodium dodecyl sulphate increased, the points moved nearer to line I and when the ratio was 1:1 by weight, the points fell upon line I. It should be noted that only the constants K_c and a_0 varied under the influence of sodium dodecyl sulphate while the constants λ_c and b_0 remained unchanged (Table II).

Hydrodynamic data indicate that the tertiary structure of GAPD became looser under the influence of sodium dodecyl sulphate when it was used in amounts up to

TABLE II

INFLUENCE OF SODIUM DODECYL SULPHATE UPON ORD PARAMETERS OF NATIVE GAPD (AT pH 8.5)

Weight ratio sodium dodecyl sulphate: GAPD	λ_c	$-K_c \times 10^{-5}$	a_0	b_0	A_{193}	A_{225}
0:1	267	100	−187	−240	416	−503
1/3:1	265	130	−220	−220	512	−634
1/2:1	262	145	−270	−230	480	−640
2/3:1	265	150	−280	−260	590	−732
1:1	264	150	−280	−250	575	−720

1/2:1 by weight; when the latter amount was not exceeded, the GAPD diffusion coefficient decreased while the molecular weight remained the same (Table III). When the quantity of sodium dodecyl sulphate introduced was increased to the ratio 1:1, a disruption of GAPD into subunits occurred. This effect is confirmed by the change in the molecular weight calculated by the Svedberg formula from the sedimentation and diffusion coefficients (Table III). For native GAPD the diffusion coefficient (D) was $4.62 \cdot 10^{-7}$ cm^2/sec, while the sedimentation coefficient (s) was 7.82 S. The molecular weight of GAPD calculated from these values for the coeffi-

TABLE III

EFFECT OF SODIUM DODECYL SULPHATE UPON HYDRODYNAMIC PROPERTIES OF GAPD (AT pH 8.5)

Preparation and conditions of measurements	$s_{20,w}$ (S)	$D_{20} \times 10^7$ (cm^2/sec)	$\bar{\nu}$	$M_{SD} \times 10^3$
GAPD	7.82 ± 0.03	4.62 ± 0.05	0.729 ± 0.001	150 ± 5
GAPD *plus* sodium dodecyl sulphate (in the weight ratio 0.1–0.5:1 with respect to the weight of the enzyme)	5.5 ± 0.5	3.65 ± 0.05	0.760 ± 0.001	152 ± 6
GAPD *plus* sodium dodecyl sulphate (in the weight ratio 0.5–1.0:1 with respect to the weight of the enzyme)	2.8 ± 0.5	4.22 ± 0.06	0.789 ± 0.001	75 ± 5

cients was 150 000; this agrees with published data[23]. Under the influence of sodium dodecyl sulphate in the ratio 1:1 the value of D changed to $4.22 \cdot 10^{-7}$ cm^2/sec while that of s changed to 2.8 S. The molecular weight changed to 75 000. Investigation of ORD data for native GAPD in the region of the bound NAD^+ band (340–400 mμ) has shown no appreciable anomalous dispersion. Similar results were obtained recently[24]. Nevertheless, a small circular dichroism was observed in the absorption band of the bound coenzyme, giving $\Delta(\varepsilon_R - \varepsilon_L) = 6 \cdot 10^{-4}$, the precision of the measurements being within $1 \cdot 10^{-4}$. This effect disappeared completely when DL-glyceraldehyde was added to the GAPD–NAD^+ complex.

If bound NAD^+ was removed from native GAPD by activated charcoal, a change in the constants of rotatory dispersion was observed (Table IV) and this indicates conformational changes in the apoenzyme. When the GAPD–$(NAD^+)_3$ complex was reconstructed by adding NAD^+ to apoenzyme, restoration of the initial values of the

TABLE IV

EFFECT OF NAD^+, IODOACETATE AND DL-GLYCERALDEHYDE ON THE ORD CONSTANTS OF APOENZYME GAPD (AT pH 8.5)

Preparation and conditions of measurements	λ_c	$-K_c \times 10^{-5}$	a_0	b_0	A_{193}	A_{225}
Native GAPD (and NAD^+ in the molar ratio 2.5:1)	268	96	−211	−200	409	−491
Apoenzyme GAPD	252	114	−279	−131	295	−443
Reconstructed complex: GAPD *plus* NAD^+ in the molar ratio 6:1	265	88	−150	−173	347	−429
GAPD (with NAD^+ in the molar ratio 2.5:1) *plus* DL-glyceraldehyde	247	125	−209	−138	261	−439
Apoenzyme GAPD *plus* iodoacetate in the molar ratio 3:1	255	130	−300	−120	376	−534
Apoenzyme GAPD *plus* iodoacetate in the molar ratio 3:1 *plus* NAD^+ in the molar ratio 5–7:1	248	130	−290	−110	284	−467

ORD constants was found (Table IV). Conformational changes in GAPD under the action of NAD^+ are connected with the NAD^+ binding at the active centre. This can be inferred from experiments with preliminary alkylation of SH groups at the active centre of the enzyme by monoiodoacetate[25]. The addition of NAD^+ to the apoenzyme first treated with iodoacetate in the molar ratio 3:1 did not cause marked changes in the constants of ORD not, consequently, in the conformation of the apoenzyme (Table IV).

Considerable conformational changes in the enzyme–coenzyme system were observed when this complex interacted with the substrate, DL-glyceraldehyde (Table IV).

The addition of DL-glyceraldehyde to GAPD solutions with bound NAD^+ in the molar ratio of 2.5:1 caused, within a few minutes, a decrease in λ_c and $-b_0$

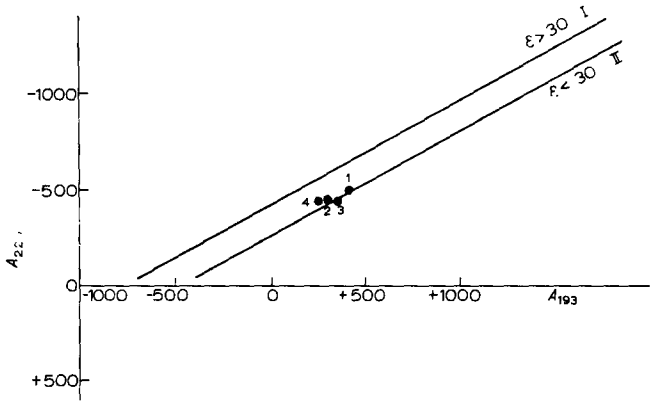

Fig. 3. Plot of SCHECHTER AND BLOUT constants A_{225} *versus* A_{193} (in a 0.1 M glycine buffer, pH 8.5). Points 1–4 (●): 1, native GAPD (with NAD^+ in the molar ratio 2.5:1); 2, apoenzyme GAPD; 3, reconstructed complex: GAPD–NAD^+ in the moler ratio 6:1; 4, native GAPD (with NAD^+ in the molar ratio 2.5:1) *plus* DL-glyceraldehyde.

(Table IV). The changes in ORD were much greater than the possible variations due to the transformation of all the NAD^+ into NADH or that of all the glyceraldehyde into glyceric acid. These changes occurred 2–3 min after DL-glyceraldehyde was introduced and were maintained for at least 1–2 h (the time during which the ORD values were checked). The conformational changes in GAPD when the coenzyme was bound or removed and when the substrate was introduced became apparent in the shift of the points along the line II (Fig. 3).

We measured the dependence of the rate of reaction (1) on pH. The results obtained are shown in Fig. 1. The curve is bell-shaped and its maximum corresponds to pH 8.5.

DISCUSSION

The rotatory properties show that GAPD as well as other dehydrogenases can be included in the first group of globular proteins according to JIRGENSON's classification[26]. This group is characterized by high λ_c and $-b_0$ values which indicate a comparatively high percentage of α-helicity (approx. 40%). The shift of the points from

line I of SCHECHTER AND BLOUT could serve to a certain degree as an indication that other ordered structures are present in GAPD besides the α-helical one. Nevertheless, experiments with sodium dodecyl sulphate enable us to come to a different conclusion. Actually, under the action of sodium dodecyl sulphate present in the ratio 0.3–0.5:1 by weight of GAPD, a displacement of the points towards line I was observed. According to data obtained by hydrodynamic methods, when such amounts of sodium dodecyl sulphate were present, a destruction of the tertiary structure occurred without its degradation into subunits. In this process sodium dodecyl sulphate did not produce changes in the α-helical structure of GAPD. Proceeding from recent data on the secondary structure of polypeptides and proteins it is difficult to explain the selective action of sodium dodecyl sulphate upon any ordered structure other than α-helices. It is most likely that sodium dodecyl sulphate destroyed the internal hydrophobic nucleus of the molecule, transferring the amino acid residues from the internal medium ($\varepsilon<30$) into the aqueous surroundings ($\varepsilon>30$). Hence, one may suggest that the shifting of the points from line I and their coincidence with the line II for several proteins is due to the influence of the local field, with dielectric constant $\varepsilon<30$, upon the optical rotation rather than to the presence of any specific ordered structure. Gradual uncoiling of the molecule, and destruction of its hydrophobic nucleus, is clearly evident in the SCHECHTER–BLOUT plot (Fig. 2).

The study of the conformation of GAPD as a function of pH showed that variation of pH was followed by changes in the tertiary and secondary enzyme structures. The dependence of α-helicity upon pH was bell-shaped. This agrees with theoretical calculations[27] for linear polyampholytes containing approximately equal numbers of acidic and basic amino acids in an alternating sequence. Indeed, GAPD contains 122 acidic amino acids and 131 basic ones. In all probability, they are situated in the molecule without forming blocks consisting only of acidic or basic amino acids.

When the pH changed from neutral to acidic or alkaline values, a shifting of the points in the SCHECHTER–BLOUT plot from line II to line I was observed. One may consider that a change in the α-helix content caused, in its turn, a change in the tertiary structure which was manifested in the uncoiling of the molecule. These data agree with those obtained in a study of the hydrodynamic properties of GAPD at various values of pH (ref. 28). A comparison of curves of λ_c and K_c as functions of pH suggests that GAPD possesses a rather labile tertiary structure and that the secondary and tertiary structures are relatively independent of each other. These conclusions are confirmed by experiments with sodium dodecyl sulphate, which changed the tertiary and left intact the secondary structure.

A comparison of the curves of α-helicity content (λ_c) and of rate of the enzymic reaction as functions of pH has shown that in this case there is no simple correlation between the catalytic properties of the enzyme and its secondary structure. Nevertheless, the maximum reaction rate was observed at pH values at which the enzyme possessed the structure with maximal α-helicity. A decrease in the α-helix content (*e.g.* on alkaline denaturation) was accompanied by a fall in the enzymic activity. In all cases, a change in the GAPD secondary structure which lowered the α-helix content was accompanied by a decrease in the rate of the catalytic reaction.

As Table IV shows, NAD^+ interacting with GAPD caused considerable changes in the GAPD structure as manifested in an increase in the α-helix content (points in the SCHECHTER–BLOUT plot moved along line II without abandoning it).

Similar results were obtained recently by FURFINE and co-workers[24]. This agrees with KOSHLAND's hypothesis of induced fit in the system enzyme–coenzyme–substrate. The coenzyme interacts with various groups in various sections of the polypeptide chains as though organizing the active centre of the enzyme. Several experiments[9] show that substrates and inhibitors are attached to dehydrogenases only after the coenzyme is bound. We obtained analogous results in studying the interactions of lactate dehydrogenase with the inhibitor oxalate[20]. In these reactions the coenzyme plays the part of the "conformational cofactor". The results of ELÖDI[28], and BOYER AND SCHULZ[29] for GAPD confirm this hypothesis. They found reversible changes in the viscosity of the enzyme solutions treated with charcoal, which adsorbs NAD^+ bound to the enzyme. There are also data on the stabilization of GAPD by coenzyme with respect to proteolysis[29]. In all probability the coenzyme acts by strengthening the enzyme structure, creating a more rigid tertiary structure and simultaneously increasing the α-helix content. Data on the variations of deuterium exchange in GAPD in the presence of NAD^+ also confirm this[21].

It is known that, when NAD^+ interacts with the GAPD molecules, a new broad absorption band appears at 340–400 mμ. We found a weak circular dichroism in this band: this was probably not an artifact since it disappeared when glyceraldehyde was added. On the other hand, the absence of anomalous dispersion in the ORD curve could have been due to insufficient sensitivity of the spectropolarimeter.

One may consider the Cotton effect in the 340–400 mμ region as due to induced optical activity appearing when NAD^+ is asymmetrically attached to the α-helix of GAPD. In this reaction glyceraldehyde interacts with that very SH group of the cysteine residue in the active centre which reacts with the nicotinamide ring in NAD^+; as a result one bond between coenzyme and enzyme is broken. This cysteine residue possibly forms a part of the α helix and the asymmetric attachment of NAD^+ to the latter induces the optical activity of coenzyme. This enables us to comprehend the disappearance of the Cotton effect in this band when NAD^+ is reduced and the bond between the coenzyme and the active centre is broken. HARRIS's suggestion[30] about the α-helical structure of the peptide which he isolated from the active centre of GAPD is in agreement with these facts.

In contrast to the studies of FURFINE and co-workers[24], our experiments on the influence of NADH upon the structure of the GAPD apoenzyme did not establish definite changes in the conformation of apoenzyme which increased the α-helix content.

When glyceraldehyde is added to GAPD which contains bound NAD^+ in the molar ratio 2.5:1, the first stage of the enzymic reaction (1) takes place; the bound NAD^+ is transformed into NADH and an enzyme–substrate complex is formed which is hydrolyzed in the absence of orthophosphate (or arsenate). Since only 3 moles of glyceraldehyde and 3 moles of NAD^+ per mole GAPD take part in the transformations, one may neglect those changes in ORD which are caused by them. The observed changes in ORD can be accounted for only by a change in the optical properties of the enzyme (and hence, of its conformation). Since the ORD changes were stable and did not disappear over a long period (1–2 h), there is little probability that they were connected with the formation of a rapidly hydrolysable enzyme–substrate complex. Changes in ORD under the action of glyceraldehyde were similar to those taking place during removal of NAD^+. One may suggest that these changes arise as

a result of the transformation of NAD^+ into NADH, whereby the influence of NAD^+ on the structure of the apoenzyme is eliminated.

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