BINDING OF SMALL MOLECULES TO LIPOAMIDE DEHYDROGENASE

H. VAN MUISWINKEL-VOETBERG

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Dit proefschrift met stellingen van

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scheikundig ingenieur, geboren te Eindhoven op 21 november 1942, is goedgekeurd door de promotor, Dr. C. Veeger, hoogleraar in de Biochemie.

De Rector Magnificus van de Landbouwhogeschool,
J. M. POLAK

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN
OP GEZAG VAN DE RECTOR MAGNIFICUS, MR. J. M. POLAK,
HOOGLERAAR IN DE RECHTS- EN STAATSWETENSCHAPPEN
VAN DE WESTERSE GEBIEDEN,
IN HET OPENBAAR TE VERDEDIGEN IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP DINSDAG 29 AUGUSTUS 1972 TE 16.00 UUR
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Aan mijn OUDERS

STELLINGEN

I

De verschuiving van een pK waarde van een essentiële groep van de eiwitketen zou een algemene verklaring kunnen zijn van het activerend effect van $NAD(P)^+$ op dehydrogenases.

Dit proefschrift

II

De terugkeer van de activiteit na pertubatie van een enzym behoeft in het algemeen geen reden te zijn voor de veronderstelling dat de native conformatie is bereikt.

Dit proefschrift

III

De uitkomst van de berekening van de percentages α-helix, β-structuur en random coil, zoals verkregen uit ORD en CD spectra, is afhankelijk van de eiwitten die als referentie worden gekozen.

YEE-HSING CHEN en JEN TSI YANG, Biochem. Biophys. Res. Com., **44** (1971) 1285

IV

De mogelijkheid om met behulp van affiniteitschromatografie informatie te verkrijgen over enzym-substraat interacties, naast kinetische gegevens, wordt momenteel nog onvoldoende onderkend.

V

Bij de replicatie van het "brome mosaic virus" is het manteleiwit noodzakelijk voor de vorming van ribonucleïnezuur component 4.

J. F. BOL, L. VAN VLOTEN-DOTING en E. M. J. JASPARS, Virology, **46** (1971) 73–85.
D. S. SHIN, L. C. LANE en P. KAESBERG, J. Mol. Biol., **64** (1972) 353–362.

VI

De acetyl-CoA synthese vanuit pyruvaat in de obligaat aerobe *Azotobacter vinelandii* behoeft niet volledig via het pyruvaat dehydrogenase complex te verlopen.

H. HAAKER, T. W. BRESTERS en C. VEEGER FEBS-Letters, **23** (1972) 160.

VII

Het door Kurtin et al. voorgestelde mechanisme inzake de waterstofoverdracht van pyridine naar lumiflavine bij excitatie is aan kritiek onderhevig.

W. E. KURTIN, M. A. LATINO en P. S. SONG, Photobiol. Photochem., 6 (1967) 247–259.

VIII

Het verschil in effect van de ionsterkte van citraat en phosphaat buffer op de NADH-lipoaat reductie van lipoamide dehydrogenase kan het gevolg zijn van de associërende invloed van het phosphaation op het enzym.

V. MASSEY, The Enzymes, 7 (1963), 275.

IX

De experimenten van ALEKSEEV en OVCHARENKO zijn onvoldoende beschreven en de toepassing van de theoretische afleiding op deze experimenten is onjuist.

O. L. ALEKSEEV en F. D. OVCHARENKO, Colloid. J. USSR, 33 (1971), 1–2

H. van Muiswinkel-Voetberg,
Wageningen, 29 augustus 1972

CONTENTS

LIST OF ABBREVIATIONS

Lipoamide dehydrogenase is the trivial name used throughout this thesis for NADH: lipoamide oxido reductase, EC 1.6.4.3, according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

BSA	bovine serum albumin
CD	circular dichroism
DCIP	2,6-dichlorophenol indophenol, oxidised form
$DCIPH_2$	2,6-dichlorophenol indophenol, reduced form
EDTA	ethylenediaminetetra acetate
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide, oxidised form
$FADH_2$	flavin adenine dinucleotide, reduced form
FMN	flavin mononucleotide
K_{ass}	association constant
K_{diss} or K_D	dissociation constant
$lipS_2$	lipoic acid, oxidised form
$lip(SH)_2$	lipoic acid, reduced form
M_{app}	apparent molecular weight
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
ORD	optical rotatory dispersion
PCMB	para-chloromercuribenzoate
SDS	sodium dodecylsulfate, sodium laurylsulfate
$s_{20,w}$	sedimentation coefficient in water at 20°
Tween 80	polyoxyethylene sorbitan mono-oleate

1. INTRODUCTION

Lipoamide dehydrogenase catalyzes under physiological conditions the reaction:
oxidized lipoic acid + NADH + H^+ ⇌ reduced lipoic acid + NAD^+. This reaction is intermediate in the oxidative metabolism of keto-acids (SEARLS and SANADI, 1960; GUNSALUS, 1954). The enzyme is also able to catalyze the oxidation of NADH by artificial electron acceptors such as 2,6-dichlorophenol indophenol (DCIP), ferricyanide (SAVAGE, 1957; MASSEY, 1960) and menadione (LEVINE et al., 1960; MISAKA and NAKANISHI, 1965). Lipoamide dehydrogenase is associated in mammalian systems and bacteria in complexes with other enzymes, these complexes e.g. the pyruvate dehydrogenase complex and the α-ketoglutarate complex, contain all the enzymes and most of the co-factors required for keto-acid oxidation (MASSEY, 1960, KOIKE et al., 1960).

REED and OLIVER (1968) were able to separate the α-ketoglutarate complex, isolated from *Escherichia coli*, into three enzymes – α-ketoglutarate dehydrogenase, dihydrolipoyltranssuccinylase and lipoamide dehydrogenase – and to reassemble the complex from these enzymes. HENNING et al. (1972) have shown that the three enzymes are present in stochiometric amouts (1:1:1).

Recently it was demonstrated by BRESTERS et al. (1972) and HAAKER et al. (1972) that the pyruvate dehydrogenase complex of *Azotobacter vinelandii* contains in addition to the three usual enzymes of the complex also phosphotransacetylase. The system is able to synthesize acetylphosphate from pyruvate under anaerobic conditions, while with the aid of soluble enzyme acetate kinase ATP can be synthesised anaerobically.

Resolution and reconstitution of the pyruvate dehydrogenase complex from *Escherichia coli* was achieved by KOIKE and REED (1963). The lipoamide dehydrogenases, isolated from both complexes, were found to be identical by physical, enzymic and immunological examination. Multiple froms of the enzymes have been found by several authors (ATKINSON et al., 1962; COHN et al., 1968; SAKURAI et al., 1969; VISSER, 1970; WILSON 1971; and STEIN and STEIN, 1972) using gel electrophoresis and ion exchange chromatography. WILSON (1971) concluded that the multiple forms are artifactual in nature due to proteolytic attack on the enzyme during the purification method. COHN et al. (1968) however suggested from electrophoresis experiments that part of the bands are derived from the α-ketoglutarate complex and the other part from the pyruvate complex. This was supported by the results of HAYAKAWA et al. (1968). Also VISSER (1970) found evidence for this suggestion.

REED and OLIVER (1968) studied the macromolecular organization of the pyruvate dehydrogenase complex and suggested from electronmicroscope data that lipoamide dehydrogenase is bound to the transacetylase in the complex in monomeric form. Isolated from the different sources, from *Escherichia coli* by KOIKE et al. (1960), from *Spinacea oleracea* by MATTHEWS and REED (1963),

from pig heart by MASSEY (1958) and VEEGER et al. (1971) and from yeast species by MISAKA and NAKANISHI (1963) and KAWAHARA et al. (1968), the physiological active species is a dimer with two moles of FAD per dimer.

High menadione and high diaphorase activity are observed with metal treated enzymes by VEEGER and MASSEY (1960), c.f. explaining the diaphorase activity as observed by STRAUB (1939) and SAVAGE (1957), which can be lowered drastically by the addition of EDTA (VEEGER and MASSEY, 1960). High DCIP activity is also observed with the monomers obtained by recombination of the apoenzyme with FAD at low temperature (KALSE and VEEGER, (1968)) and by freezing (VISSER, 1970) of the fully reduced enzyme or of low concentrations of enzyme.

Upon reduction of the enzyme with NADH or reduced lipoic acid a S-S bridge is reduced and two additional sulfhydryl groups become available as observed by titration of sulfhydryl groups (MASSEY and VEEGER, 1960; SEARLS, 1960). This disulfide was demonstrated to be involved in catalysis and to be in the vicinity of the flavin. Lipoamide dehydrogenase becomes fully reduced by two moles of dithionite (four electrons). Since flavin itself can accept only two electrons a second electron acceptor is implicated (MASSEY et al., 1960), e.g. the S-S bridge. After blocking the dithiol, produced by reducing the enzyme with one mole NADH per mole flavin, the reoxidized enzyme becomes fully reduced by the addition of another mole of NADH per mole flavin without intermediate formation of the two electron reduced state, the semiquinone, of the flavin (STEIN and STEIN, 1972). On the other hand VEEGER et al., (1966,) observed transit intermediates.

Except this disulfide in the active center the pig heart enzyme contains another disulfide and six sulfhydryl groups per flavin (PALMER and MASSEY, 1962). In the native enzyme two sulfhydryl groups react instantaneously (VEEGER and MASSEY, 1962) when p-chloromercuribenzoate was used as titrant. The observation that after reaction with PCMB for 24 hours seven SH-groups were blocked and flavin dissociated from the protein led to the suggestion that the non-covalent association of the flavin with the protein involved a sulfhydryl group (MASSEY, 1963). In addition amperometric titration with phenylmercuric acetate yielded 4-5 sulfhydryl groups in the native enzyme (PALMER and MASSEY, 1962). CASOLA and MASSEY (1966) concluded that also with phenylmercuric acetate two SH-groups react instantaneously and the others upon denaturation. In the initial phase of mercurial treatment of the native enzyme the DCIP activity is enhanced but the active center disulfide is not involved because in the DCIP reaction the flavin oscillates between the fully oxidized and the fully reduced form (CASOLA and MASSEY, 1966). With iodoacetamide also two sulfhydryl groups are rapidly alkylated with no influence on the artificial menadione activity (MISAKA and NAKANISHI, 1965). The lipoate activity of pig heart enzyme is inhibited after blocking of four SH-groups per mole of enzyme with PCMB (CASOLA and MASSEY, 1966). The enzyme isolated from bakers yeast contains three SH-groups per flavin (MISAKA, 1966). The menadione activity is inhibited by NAD^+. PCMB stimulates the menadione activity which decreases upon the addition of NAD^+. This inhibition by NAD^+ was non-competitive.

Upon incubation for a few hours with N-(2,2,5,5 tetramethyl pyrrolidinyl-1-oxyl)maleimide two sulfhydryl groups are blocked in the pig heart enzyme while the lipoate activity has declined to 70% and the DCIP activity is stimulated (GRANDE and MÜLLER, 1972). WILLIAMS et al. (1971) found conflicting results upon alkylation of the pig heart enzyme with iodoacetic acid, eight sulfhydryl groups are found but reduction did not increase the number of sulfhydryl groups. STEIN and STEIN (1972) observed loss of lipoate activity upon alkylation of the native oxidized pig heart enzyme by iodoacetamide.

CASOLA et al. (1966) correlated the loss of lipoate activity and the increase in DCIP activity upon Cu^{++}-treatment with the oxidation of sulfhydryl groups. Oxidation of two SH-groups to a disulfide caused extensive changes in catalytic properties without changes of protein and flavin fluorescence during the initial stage of the Cu^{++}-treatment. GRANDE and MÜLLER (1972) showed that the oxidation of two SH-groups to a disulfide upon Cu^{++}-treatment involved one of the rapidly reacting SH-groups. Other metals are known to bind to the catalytic dithiol of the reduced enzyme (VEEGER and MASSEY, 1960; MISAKA and NAKANISHI, 1966b; STEIN and STEIN, 1972). Mercurials, Cu^{++} and Cd^{++} destabilise the 'semiquinone', the two electron reduced form of the enzyme which is EPR inactive (SEARLS and SANADI, 1960), and further reduction to the fully reduced form of the flavin occurs. MISAKA and NAKANISHI (1966a) concluded from the absence of the long wavelength band in the spectrum of the PCMB-treated enzyme upon reduction that a free SH-group participates in the combination of the enzyme with NAD^+. The long wavelength band being due to a charge transfer complex between NAD^+ and $FADH_2$ (MASSEY and PALMER, 1962).

Replacement of the amide side group of the pyridinium ring of NAD^+ by an acetyl or aldehyde group results in loss of activity. Only replacement by thioamide gives normal activity (MASSEY, 1963). Also the aminogroup of the adenine moiety of NAD^+ is important as replacement by a hydroxylgroup gives very low activities.

Reactivity is also influenced by the nature of the lipoylderivative. Shift of the dithiolane ring to the carboxyl group yields derivatives that are not able to form the enzyme-substrate complex (GOEDDE et al., 1963). With derivatives possessing a charged group, the turnover number increases the further the charged group is separated from the dithiolane ring and is even larger when the charged group is effectively neutralised in a zwitterion structure as lipoyl-ε-lysine (MASSEY, 1963). Structures which are unionized in the oxidized form show the highest activity, this suggest that a anionic charge on the substrate hinders the approach to the enzyme (MASSEY, 1963). Closer structural resemblance to the natural bound substrate is according to NAWA et al. (1960) the reason why for instance ε-N-(d-L-lipoyl)-L-lysine is a better substrate than lipoamide which in turn is better than lipoic acid. Lipoic acid, the natural substrate, is bound to an ε-lysine in the complex, to a lysine residue of the transacetylase in the case of the pyruvate-dehydrogenase complex (REED and OLIVER, 1968). After isolation lipoamide dehydrogenase from *Escherichia coli* the soluble substrate lipoic acid was shown

to be bound to an ε-amino of a lysine residue (NAWA et al., 1960).

The non-covalently bound flavin molecule is found to play the most important role in the stabilisation of the tertiary protein structure (VISSER, 1970). THEORELL and NYGAARD (1954) suggested that the 3-position of the flavin molecule was involved in the binding to the protein.

VISSER observed binding of 3-substituted flavins with partial restoration of the DCIP activity, but found that modifications of the ring diminished the affinity of the protein for the flavin compound. The holoenzyme is not FMN sensitive, however the recombined enzyme is FMN sensitive yielding an inactive enzyme. This sensitivity decreases with time. VISSER (1970) concluded from these results that the interactions between the protein and the flavin are based on multiple binding forces as already suggested by several authors for the binding of FAD to flavoproteins (THEORELL and NYGAARD, 1954; THEORELL, 1958; WALAAS and WALAAS, 1956). BRADY and BEYCHOK (1969) reported a low affinity of FMN for the apoenzyme and suggested that this nucleotide has a stabilising influence on the apoenzyme during their procedure of preparing apoenzyme.

They found for apoenzyme prepared in the presence of FMN a circular dichroism spectrum coincident with that of the native holoenzyme, with a calculated amount of α-helix of 35%. The α-helix content is influenced by the contributions of FAD (MILES and URRY, 1968). The circular dichroism curves in the 190–240 nm region do not change upon the addition of substrate, NADH or dihydrolipoate (BRADY and BEYCHOK, 1969).

KALSE and VEEGER (1968) determined the molecular weight of the apoenzyme, 52.000, half that of the holoenzyme, while the apoenzyme prepared according to BRADY and BEYCHOK (1969) was found to have a molecular weight of 100.000 indicating that the latter is a dimer (MASSEY, private communication).

Dissociation can also be achieved by urea treatment of the enzyme (MASSEY et al., 1962). VISSER (1970) presented evidence that freezing at low concentrations or reduction by four electrons favors dissociation. These dissociation phenomena led to the conclusion that the two S-S bridges are both intramolecular and not connecting both subunits as suggested by MASSEY et al. (1962). WILLIAMS and ARSCOTT (1972) determined the sequence of a peptide containing the active center S-S bridge for the pig heart enzyme as well as for the *Escherichia coli* enzyme.

Pig heart:

Glu-Thre-Leu-Lys-Gly-Thre-Cys-Leu-Asn-Val-Gly-Cys-Ileu-Pro-Ser-Ala-Leu

S——————S

Escherichia coli:

Val-Cys-Leu-Asn-Val-Gly-Cys-Ileu-Pro-Ser-Lys

S——————S

MASSEY and VEEGER (1961) proposed a reaction mechanism for the pig heart enzyme. Upon reduction by NADH or dihydrolipoate two electrons are accepted, yielding the red semiquinone intermediate. It was proposed that an electron

of the flavin is in interaction with the electron of the sulfur radical, forming an EPR inactive species. Reduction of the enzyme by excess NADH in the presence of NAD-ase results in the four electron reduction of the enzyme. NAD^+ prevents four electron reduction and stabilises the semiquinone state. STEIN and CZERLINSKI (1967) reported the complex formation of the oxidized enzyme with NAD^+. They found evidence for complexes of more than one mole NAD^+ per mole FAD at concentrations of NAD^+ higher than 5 mM.

Motivation for this investigation was the observation of REED and OLIVER (1968) that in the complex the enzyme was bound in a monomeric form.

The existence of a dimer-monomer equilibrium was looked for and the factors influencing this equilibrium investigated.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. *Enzymes*

The purification method used for lipoamide dehydrogenase from pig heart has been described elswhere (VEEGER and VISSER, 1970). The procedure for the preparation of the apoenzyme and the recombination of the apoenzyme with FAD is described by KALSE and VEEGER (1968). The removal of the flavin is achieved by an acid $(NH_4)_2SO_4$ treatment, according to the method used by STRITTMATTER (1961) for cytochrome b_5 reductase. BRADY and BEYCHOK (1969) reported the preparation of the apoenzyme by dialysis against guanidine hydrochloride at neutral pH in the presence of FMN and mercaptoethanol. The difference between the two preparations is that the acid treatment yields a monomeric apoenzyme while the guanidine treatment yields a dimeric apoenzyme (MASSEY, private communication).

With the method used here the individual apoenzyme preparations, which are obtained, may rather vary. The flavin content amounts to approximately 5% or less, some of which is not enzyme-bound. The rest activity with lipoate is 0.3–3% while the DCIP activity varies between 80 and 300% of the original activity of the holoenzyme.

Cu^{2+}-treated enzyme was obtained by incubating the enzyme with a $CuSO_4$ solution (8 mole Cu^{2+} per mole FAD, CASOLA and MASSEY, 1966). The treatment was stopped by the addition of excess EDTA.

2.1.2. *Reagents*

NAD^+, FAD, FMN, lipoic acid, bovine serum albumin, sodium dodecylsulfate and Tween 80 were obtained from Sigma Chemical Co. NADH and alcohol dehydrogenase were from Boehringen and Söhne. DCIP came from the British Drug House. Ovalbumin, acrylamide and N-N'-methylenebisacrylamide were purchased from Kochlight. Blue dextran 2000 and Sephadex G-200 were from Pharmacia. Amido Schwartz was obtained from Hartman-Leddon Co, Philadelphia. Ammonium persulfate and N,N,N'N'-tetramethylethylenediamine from E. C. Compagny, Philadelphia. Ethanolamine and bromocresolpurple were obtained from E. Merck AG and carrier ampholytes from LKB-Produkter AB. Lipoamide was synthesised according to REED et al. (1959).

2.2. METHODS

2.2.1. *The enzymatic assay of lipoamide dehydrogenase*

The enzyme activity of the NADH-lipoate reaction:

$$NADH + H^+ + lipS_2 \rightleftharpoons NAD^+ + lip(SH)_2$$

is assayed spectrophotometrically by recording the decrease in absorbance at

340 nm. To a spectrophotometer cuvette which is thermostated at 25° is added: 2.5 ml citrate buffer (1 M, pH 5.65); 0.1 ml bovine serum albumin (2% w/v in 30 mM EDTA); 0.1 ml lipoate (20 mM); 0.03 ml NAD^+ (10 mM solution in bidest); 0.03 ml NADH (10 mM in bidest, freshly prepared every day and kept on ice) and bidest to a final volume of 3 ml. The reaction is started by adding enzyme in an appropriate dilution giving an initial decrease in absorbance at 340 nm that does not exceed 0.2 per minute.

The diaphorase activity is also determined spectrophotometrically by recording the reduction of DCIP at 600 nm:

$$NADH + H^+ + DCIP \rightarrow NAD^+ + DCIPH_2$$

To a spectrophotometer cuvette, which is thermostated at 25°, is added: 0.5 ml sodium phosphate buffer (0.3 M, pH 7.2); 0.1 ml bovine serum albumin (2% w/v in 30 mM EDTA); 0.12 ml DCIP (1 mM in bidest); 0.03 ml NADH (10 mM in bidest, freshly prepared every day and kept on ice) and bidest to a final volume of 3 ml. The reaction is started by adding an amount of enzyme which gives an initial decrease in extinction at 600 nm that does not exceed 0.25 per minute. The activities are based on the initial rates. The activity with DCIP is obtained by multiplication of the specific activity by a factor 100 (SAVAGE, 1957).

Activities are generally expressed in % activity with respect to those of the holoenzyme.

2.2.2. *Gel-filtration*

Sephadex G-200 columns were calibrated using alcohol dehydrogenase, bovine serum albumin and cytochrome-c as standard proteins. Void volumes were determined with blue dextran-2000. The elution buffers used all contained 0.3 mM EDTA in 30 mM phosphate buffer pH 7.2 when not otherwise stated. Fractions of 3 ml were collected with a LKB fraction collector. The absorption pattern of the elution solution was registrated with a 8300 A Uvicord 11 at 260 nm.

2.2.3. *Ultracentrifugation*

Sedimentation patterns were obtained using an M.S.E. analytical ultracentrifuge. Rotor speed used was 51500 revolutions per minute. The experiments were performed at 17°. Photographs were taken at at least seven different times. Molecular weights were determined using the Svedberg relationship. In this relationship the proteins are assumed to be globular and not to change in shape or size during the experiments, which is in the case of the SDS experiments probably not justified. A difference in specific volume of 0.02 will give rise to a deviation of 10% in the value found for the molecular weight. Ideal behaviour is also assumed in this relationship.

Sedimentation coefficients were determined grafically.

2.2.4. *Light-scattering*

Light scattering data were obtained with a Cenco-TNO apparatus at room temperature. Measurements were kindly performed by Mr. VAN MARKWIJK

(N.I.Z.O., Ede, The Netherlands). The molecular weight has been calculated according to the relation:

$$R = \frac{i}{I_0} = \frac{Kc}{1/M + 2Bc + 3Cc^2 + \ldots} \; cm^{-1}$$

(TANFORD, 1961) in which the optical constant K is defined as

$$K = \frac{2\pi^2 n_0^2 (dn/dc)^2}{N\lambda^4} \; cm^2 g^{-2}$$

dn/dc = refractive increment = 0.176 $cm^3 g^{-1}$ for lipoamide dehydrogenase in 30 mM phosphate buffer pH 7.2 with 0.3 mM EDTA (VISSER, 1970)

n_0 = index of refraction of the solvent

λ = wavelength of the light used, 5460 Å

$\frac{i}{I_0}$ = ratio of the intensities of the light scattered under the angle and of the incident beam. Benzene was used as a standard.

The constant K amounts 2.57 10^7 $cm^2 g^{-2}$. The samples were filtered before use with a 100 mμ filter and the values were corrected for the contributions of the solvent.

2.2.5. *Electrofocusing*

Electrofusing measurements were performed with a LKB 8100 Ampholine Electrofocusing Equipment. The cathode solvent used was 0.2 ml ethanolamine in 10 ml bidest, as anode solvent 0.3 ml phosphoric acid in 14 ml bidest with 12 g sucrose. The gradient was formed from two components. The heavier component consisted of 3.7 ml carrier ampholite 5–8 in 42 ml bidest with 28 g sucrose and the lighter one consisted of 1.3 ml carrier ampholite 5–8 in 60 ml bidest with 0.15 ml lipoamide dehydrogenase (30 mg/ml). The 110 ml LKB column was used. The experiments were performed at 9 °. The voltage applied was increased during the experiment, at the start the power was 0.2 Watt, during the experiment it never exceeded 1.2 Watt. Fractions of 1 ml were collected and the pH determined.

2.2.6. *Circular Dichroism*

CD spectra were recorded on a Jouan Dichrographe II. Cells ranging from 100 to 0.1 mm were used. The cell compartment was flushed with dry nitrogen. Special care was taken to place the cell in the right position upon recording in the far ultra-violet region. It was checked by the method described by MILES and URRY (1968) that at high optical density no shift in baseline occurred.

2.2.7. *Difference spectroscopy*

Difference spectra were recorded on a Cary 14 using tandem cells by the method as described by LASKOWSKI et al. (1960). Only in the case of difference spectra with detergents added to the enzyme single cells were used since micelle formation occurred in the reference buffer cell to which detergent had to be added.

2.2.8. *Optical Rotatory Dispersion*

ORD measurements were made with a Jasco ORD/UV-5. Cell length varied from 100 to 1 mm. The data were partially analysed in terms of the empirical Moffitt-Yang equation (MOFFITT and YANG, 1956, MOFFITT, 1956):

$$[m'] = \frac{\alpha\, MRW}{c\, l} = \frac{a_0\, \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\, \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad \frac{degrees\ cm}{decimole}$$

in which the mean residue weight (MRW) = 115
λ_0 = 216 nm
α = the rotation at that wavelength in degrees
c = concentration of the protein in g/ml
l = cell length in decimeters
and partially in terms of the specific rotation (α) at 250 nm. Originally b_0 was considered to be principally a function of the helix backbone, independent of side chain interactions and environmental influences while a_0 should represent the rotation irrespective of the helix and the interactions within the helix (MOFFITT and YANG, 1956). Due to the presence of the flavin chromophore, which shows strong optical activity in the 200–240 nm region (MILES and URRY, 1968, SIMPSON and VALLEE, 1966, LISTOWSKY et al., 1966) this interpretation cannot be correct for this protein. The parameters a_0 and b_0 were determined grafically. Data were not corrected for refractive index.

2.2.9. *Fluorescence*

The fluorescence measurements were performed with a Hitachi Perkin Elmer MP 2A fluorospectrophotometer which was equipped with a thermostated cuvette holder. The excitation and emission spectra are corrected for scatter of the solvent and variations in the intensity of the 150 W Xenon-light source.

If two fluorescence spectra of the protein are made coincident at 375 nm by multiplication by an appropriate factor, the difference spectrum obtained is that of the tyrosine contribution, if the spectra substracted are obtained by excitation at 280 nm and 292 nm. If the spectra substracted are obtained by excitation at 292 and 297 nm the difference spectrum represents the contributions of the pertubed tryptophans (WEBER and YOUNG, 1964).

2.2.10. *Viscosity*

Viscosity measurements were made with Oswald viscosimeters. Flowtimes measured were between 400 and 450 seconds. The waterbath was thermostated within 0.05°. Solutions were centrifuged and filtered before use. Experiments were performed with two viscosimeters with about the same flowtime.

Results were expressed as kinematic viscosity which was calculated from the relation:

$$v = t\, c\text{–}1/t \text{ centistokes}$$

in which
c = constant, dependent on the properties of the viscosimeter
t = flowtime in seconds

2.2.11. *Fluorescence polarisation*

Fluorescence polarisation measurements were performed with an instrument built by DE KOK identical to the one described by WEBER (1956). As excitation wavelength for FAD 365 nm was chosen. The polarisation of the fluorescent light emitted is defined by

$$p = \frac{I_{vV} - I_{hV}}{I_{vV} + I_{hV}}$$

in which

I_{vV} = vertical polarised emission vector obtained by excitation with the vertical excitation vector

I_{hV} = horizontal polarised emission vector obtained by excitation with the vertical excitation vector.

2.2.12. *Determination of the number of binding sites from spectrophotometric data*

Determination of the number of binding sites can be done by employing the equation derived by KLOTZ (1946) for binding of ions by proteins. For n independent binding sites this equation is

$$\frac{[P]}{[I]_{bound}} = \frac{K'_{diss}}{n([I]_{total} - [I]_{bound})} + \frac{1}{n}$$

in which

[P] = concentration of protein in M

$[I]_{total}$ = total concentration of ion in M

$[I]_{bound}$ = concentration of ion bound to the protein in M

K'_{diss} = intrinsic dissociation constant in M

n = number of binding sites

By assuming that $[I]_{bound}$ is lineairly related to the difference in absorbancy at a certain wavelength, the equation becomes, rearranged

$$\frac{[I]_{total}}{\Delta A/\Delta A_{max.}} = \frac{K'_{diss}}{(1-\Delta A/\Delta A_{max.})} + n\,[P]$$

in which

$\Delta A_{max.}$ = difference in absorbancy at saturating conditions

ΔA = difference in absorbancy at that particular ion concentration

The number of binding sites, n, and K'_{diss} can be determined grafically (STOCKELL, 1959).

2.2.13. *Dialysis*

Dialysis against NaCl solutions pH 5.7 was continued for 72 h. Every four hours the NaCl solution was removed and dialysis was continued in 300–400 ml of fresh NaCl solution. All solutions contained 0.3 mM EDTA.

3. DISSOCIATION OF LIPOAMIDE DEHYDROGENASE

3.1. INTRODUCTION

NADH-lipoamide oxidoreductase has been isolated from a number of sources, for instance pig heart (STRAUB, 1939, SAVAGE, 1957 and MASSEY, 1961), dog fish liver (CHANNING et al., 1962), *Escherichia coli* (HAGER et al., 1953 and KOIKE et al., 1963) *Mycobacterium tuberculosis* (GOLDMAN, 1960), *Saccharomyces* species (WREN and MASSEY, 1965) and from *Spinacea oleracea* (BASU and BURMA, 1960, MATTHEWS and REED, 1963). The enzyme as isolated from the different sources appears to be a dimer. In the case of the enzyme isolated from pig heart the association between the peptide chains is of hydrophobic nature (KALSE and VEEGER, 1968). This means that under certain circumstances it must be possible to dissociate the enzyme reversibly. Light-scattering experiments have shown that the holoenzyme is able to dissociate upon dilution (VISSER, 1970). The monomer-dimer equilibrium is pH dependent.

A protein has a certain conformation because that state is the thermodynamically preferred one under that specific condition. It is possible that different conformations are obtained under variable conditions and that the enthalpy and entropy barriers for the change of conformations into each other is high so that conversion is slow, like for example the conversion of α into γ chymotrypsin (LUMRY and BILTONEN,1969). Also aggregation at high temperatures may cause such effects that dissociation at low temperatures can only be achieved in acetic acid, urea, etc., followed by dilution and dialysis (LUMRY and BILTONEN, 1969).

The conformation of proteins can be influenced by several factors. In the literature a lot of examples are given. Most studies are concerned with thermal and solvent pertubations, ion and neutral salt binding. Neutral salts react generally with charged residues located on the surface of the molecule or with dipolar groups. Site binding interactions are characterized by high association constants and are strongly dependent on the protein concentration (VON HIPPEL and SCHLEICH, 1969). Lyotropic or Hofmeister type effects of neutral salts on macromolecular structures seem sometimes to be reflected in the activity of enzymes. FRIDOVIC (1963) studied the inhibition of acetoacetic acid decarboxylase by various anions and showed that the inhibition can be explained by a very tight binding of the various anions to a cationic site at the active site. The inhibition is dependent on the pH. Also serum albumin binds small ions. The binding increases with the polar character of the ions. In this case tryptophan is suggested to be involved in the binding.

Effects of neutral salts on macromolecules may also have influence on the protein-protein interactions. The effectiveness of the salts follows a lyotropic serie, one end favors association, the other dissociation. For example, muscle glyceraldehyde, 3-phosphate dehydrogenase is dissociated into a 3.2 S species by KCl and associated into a 4.4 S species by $(NH_4)_2SO_4$ (CONSTANTINIDES and

DEAL, 1970). Also dissociation of haemoglobin in αβ-dimers by salts is shown by various authors, with 1 M $CaCl_2$ by KAWAHARA et al. (1965) and with 2 M NaCl by BENESCH et al. (1964).

Solvent pertubation is commonly used to show the exposed or buried state of amino acid residues side chains in the conformation of macromolecules and most studies are not concerned with the origin of the spectral changes. However pertubing solvents can affect the conformation of proteins, it can have influence on the state of aggregation of the protein. Preferential interaction of the pertubant with one of the conformational states can shift the equilibrium in a certain direction. Glutamate dehydrogenase shows head to tail polymerisation to very large polymers upon the binding of twelve moles of tolueen per mole of active enzyme, the hexamer (SUND, 1972).

As dissociation of lipoamide dehydrogenase upon dilution as observed by VISSER (1970) was variable from preparation to preparation, it was of interest to investigate whether the monomer-dimer equilibrium could be influenced by neutral salts or solvents of low dielectric constant.

3.2. RESULTS

3.2.1. *Dissociation upon dilution*

Upon dilution the lipoate activity of lipoamide dehydrogenase decreases. Light-scattering experiments show that $\overline{M}_{app}$ decreases in accordance with the lipoate activity (VISSER, 1970). The dissociation is pH dependent, at pH 8.2 no decrease in $\overline{M}_{app}$ is found. Attempts to shift the equilibrium to the dimer side by concentrating a diluted enzyme solution were unsuccesfull, while the activity of the dimer was not affected by the concentration procedure (centrifuging overnight at 100,000 g or precipitation with $(NH_4)_2SO_4$).

There was also a large difference between preparations. Sometimes the lipoate activity did not decrease even at extreme dilution. However freezing of these preparations for long times (several months) made return the ability to dissociate. Such an effect could be explained by changes in the pH of the solution during the freezing process, as has been observed for lactate dehydrogenase (CHILSON et al., 1965).

The velocity of the conversion could be determined by the presence of certain ions. During the isolation of the enzyme a heating step, 5 minutes at 70° in high phosphate concentration, is included. Due to the hydrophobic character of the subunit interaction this step will favor association. Therefore dissociable enzyme was heated again during 5 minutes at 70° in 0.2 M phosphate buffer pH 7.2. The lipoate activity increases, but the ability to dissociate, although less, did not completely disappear. Heating without phosphate did not lead to reactivation, while in the absence of phosphate the enzyme is more heat-sensitive.

As phosphate has influence on the activity of the enzyme (VEEGER and MASSEY, 1961) the non-dissociable enzyme was dialysed against NaCl solutions of different ionic strengths at pH 5.6 because dissociation is prevented at high pH

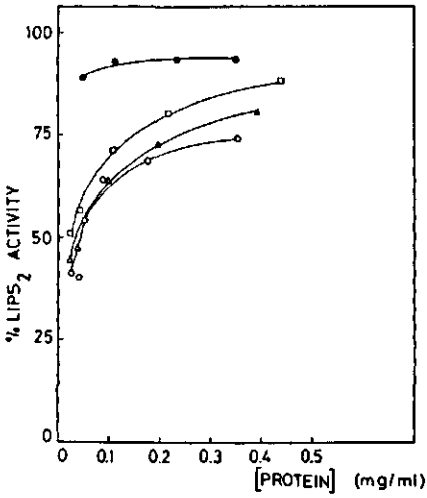


FIG. 1. Effect of dialysis against NaCl on the lipoate activity of diluted lipoamide dehydrogenase. Enzyme (10 mg/ml) was dialysed against NaCl solutions of the ionic strength indicated, pH 5.6 containing 0.3 mM EDTA during 72 h with ten changes of solution. The activity was measured as given in the methods. When precipitation occurred the solution was centrifuged and the supernatant was used for the activity determination. Temperature 25°. ●—●, Control in phosphate buffer, 30 mM pH 7.2 0,3 mM EDTA; ○—○, I = 0.01; △—△, I = 0.04; □—□, I = 0.10.

(VISSER, 1970). Fig. 1 shows the decrease of the lipoate activity upon dilution in NaCl solutions of different ionic strengths after dialysis against the same solutions. However also a slight decrease in activity was observed upon dilution when the enzyme was dialysed against 30 mM phosphate buffer. At high ionic strength of the NaCl solution a higher activity is obtained at high protein concentration compared with the activity at low ionic strength. In these experiments the protein became insoluble after extensive dialysis against solutions of low ionic strength (I: 0.01 and I: 0.02). The precipitate was dissolved in phosphate buffer pH 6.8 and the activity compared with the lipoate activity in the supernatant (Fig. 2). The dissolved precipitate contained aggregates, as was determined by light-scattering and Sephadex G-200 chromatography, with molecular weight higher than 800,000.

It was of interest to see whether the decrease in lipoate activity after dialysis was due to dissociation of the enzyme. On Sephadex G-200 the molecular weight of the enzyme in 30mM phosphate buffer pH 7.2 was determined, a molecular weight of 100,000 was found and compared with the molecular weight of a NaCl-dialysed enzyme, with 0.05 M NaCl pH 5.6 as elution buffer. When 0.5 ml of an enzyme solution containing 0.8 mg/ml was brought on the column one main peak was observed with a molecular weight of 65,000–70,000. With a two times higher protein concentration again one peak was observed but with a molecular

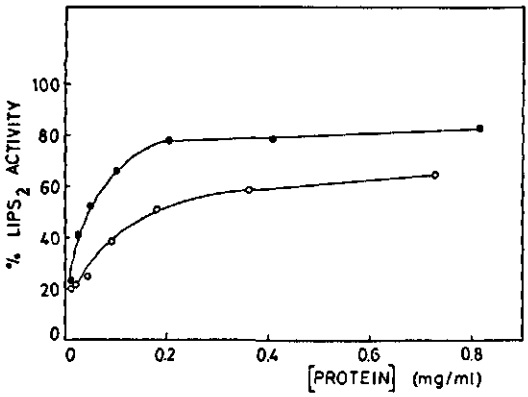


FIG. 2. Comparision of the lipoate activity of the supernatant and of the precipitate of an enzyme solution dissolved in phosphate buffer I = 0.02, pH 6.8. Enzyme, 10 mg/ml, dialysied for 72 h with ten changes of NaCl solution I = 0.02, pH 5.6 containing 0.3 mM EDTA. Temperature 25°. ●—●, supernatant; ○—○, dissolved precipitate.

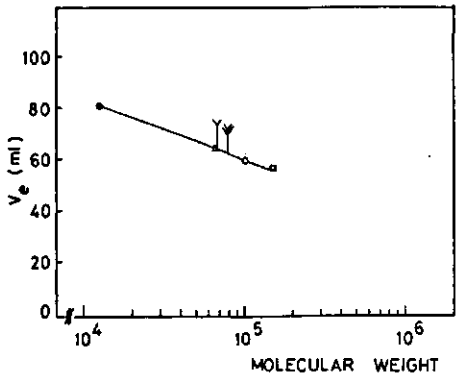


FIG. 3. Dependency of the molecular weight of the NaCl-dialysed enzyme on the protein concentration. The column was calibrated with cytochrome c (●), BSA (△), alcohol dehydrogenase (□) and lipoamide dehydrogenase holoenzyme (○). Arrow indicates value found for a solution of 0.8 mg/ml of NaCl-dialysed enzyme, double arrow value for a solution of 1.6 mg/ml of NaCl-dialysed enzyme. Temperature 25°.

weight of 75,000–80,000 (Fig. 3). In both cases the peaks showed a tailing to lower molecular weight. The specific activity of the enzyme in the peaks was in the latter experiment higher than in the first.

It was tried with the NaCl-dialysed enzyme to shift the equilibrium to the dimer side by several methods. As NAD^+ stabilizes the enzyme (chapter 5) addition of NAD^+ to the diluting buffer was tested for an effect on the lipoate activity (Fig. 4). NAD^+ did show some increase in the lipoate activity, however it cannot prevent dissociation completely. Temperature has no influence on the extent of dissociation; neither in the absence nor in the presence of NAD^+. Heating at 70° during 5 minutes in high phosphate concentrations has the same influence on the NaCl-dialysed enzyme as on the phosphate enzyme. When the enzyme was first dialysed against 30mM phosphate buffer pH 8.2 and afterwards diluted in NaCl solutions pH 5.6 the effects were considerably diminished (Fig. 5). Dilution of NaCl-dialysed preparations in phosphate buffer of high ionic strength did give protection against inactivation but the preparation could be further activated by heat treatment in high phosphate concentration.

As a part of the enzyme precipitated during extensive dialysis against NaCl solutions pH 5.6 of low ionic strength (I: 0.01 or 0.02) it was of interest to see if precipitation occurred at the iso-electric point. With iso-electrofocusing the iso-ionic point is obtained instead of the iso-electric point, but the difference between these is believed not to be more than a few tenths of a pH unit (TANFORD, 1962). The electrofocussing pattern shows six bands, in agreement with the electrophoresis pattern (VISSER, 1970). In all the six bands lipoate activity could be demonstrated. From these results it seems likely that the precipitation is due to a

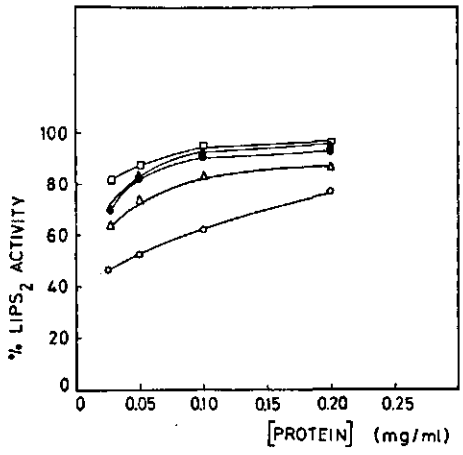


FIG. 4. Influence of NAD^+ and phosphate on the lipoate activity of lipoamide dehydrogenase. Enzyme, 10 mg/ml, dialysed against 0.05 M NaCl, pH 5.6 containing 0.3 mM EDTA for 72 h and diluted in the buffer indicated. Temperature 25°. ○—○, NaCl, I = 0.05, pH 5.6; △—△, NaCl, I = 0.05, pH 5.6 containing 1 mM NAD^+; ●—●, phosphate buffer, I = 0.07, pH 7.2; ▲—▲, phosphate buffer, I = 0.05, pH 5.6; □—□, phosphate buffer, I = 0.07, pH 7.2 containing 1 mM NAD^+.

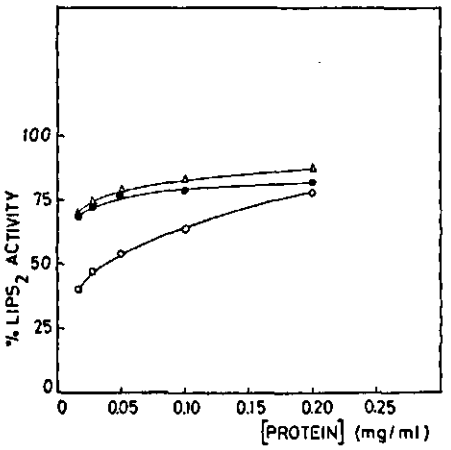


FIG. 5. Influence of heating lipoamide dehydrogenase in high phosphate concentration on the activity of lipoamide dehydrogenase. An enzyme preparation, 10 mg/ml, was dialysed against 0.05 M NaCl, pH 5.6, containing 0.3 mM EDTA during 72 h or against phosphate buffer, I = 0.07, pH 8.2, containing 0.3 mM EDTA and diluted in the buffers indicated. Temperature 25°. ○—○, dialysed and diluted in NaCl, I = 0.05, pH 5.6; △—△, dialysed and diluted in NaCl, I = 0.05, pH 5.6 after 5 minutes at 70° in 0.2 M phosphate buffer pH 7.2; ●—● dialysed against phosphate buffer, I = 0.09, pH 8.2 and diluted in NaCl, I = 0.05, pH 5.6 after 5 minutes at 70° in 0.2 M phosphate buffer pH 7.2.

decrease in solubility of the enzyme in solutions of low ionic strength. Further support comes from the observation that the same results are obtained in the presence of KCl.

3.2.2. *Effects of pertubants*

When the enzyme dissociates upon dilution a tryptophan becomes more exposed to the solution (chapter 4). Pertubation of amino acid residues can also be achieved in solutions of low dielectric constant. Fig. 6 shows the influence on the lipoate activity of the enzyme at different protein concentrations upon lowering the dielectric constant of the enzyme solution by the addition of different solvents. At low temperatures the effects of pertubants are markedly larger (see Fig. 6 and 7). If the decrease in lipoate activity is due to the decrease in dielectric constant of the solution the same effect must be observed by increasing the volume percentage of one of the different solvents. Fig. 7 shows the decrease in lipoate activity at increasing dioxan and 2-chloroethanol concentrations. The decrease in lipoate activity is time-dependent. The first one to two hours the lipoate activity remains at the initial level or even slightly increases while afterwards it starts to decline (Fig. 8). The decrease in activity is not due to denaturation as can be concluded from ORD measurements, since $[m']_{233}$ does not change during the time that the lipoate activity under the influence of dioxan decreases from 100 to 30%. Increase of the phosphate concentration in the solution shows a higher

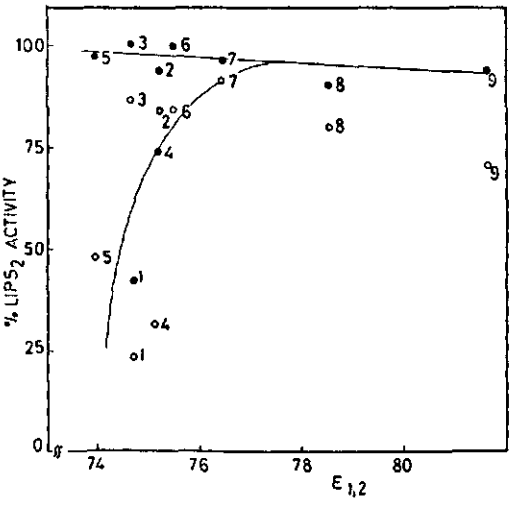


FIG. 6. Effect of different solvents on the lipoate activity of lipoamide dehydrogenase. Enzyme was dissolved at the concentrations given in a 20% solution of the solvent indicated in 30 mM phosphate buffer pH 7.2. The activity was measured in the NADH-lipoate reaction of an aliquot from the mixture without prior dilution. Temperature 25°. ●—●, 0.14 mg/ml; ○—○, 0.014 mg/ml.

1. Dioxan; 2. Aceton; 3. Ethanol; 4. 2-Chloroethanol; 5. Methanol; 6. Glycol; 7. Glycerol; 8. Water; 9. Formamide;

ε was calculated according to $\varepsilon = \frac{x_1 \varepsilon_1 + x_{water} \varepsilon_{water}}{x_1 + x_{water}}$

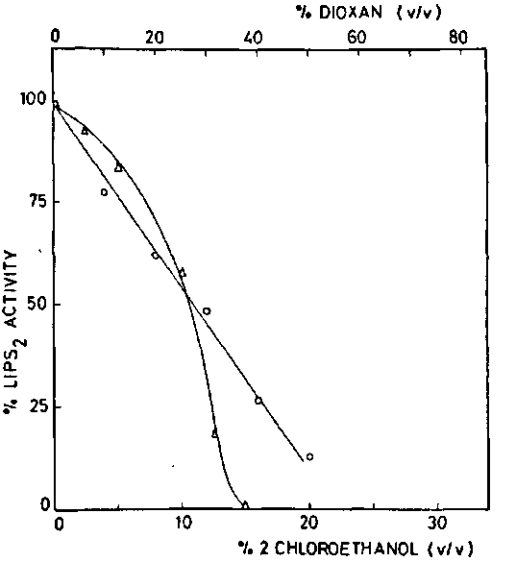


FIG. 7. Influence of dioxan and 2-chloroethanol on the lipoate activity of lipoamide dehydrogenase. The activity of enzyme, 0.3 mg/ml, was measured as given in the methods. The enzyme was dissolved in 30 mM phosphate buffer pH 7.2 containing 0.3 mM EDTA with increasing volume percentage of dioxan and 2-chloroethanol. ○—○, dioxan; △—△, 2-chloroethanol. Temperature 0°.

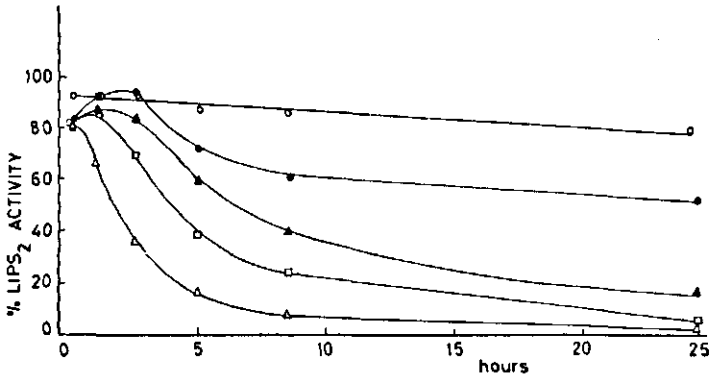


FIG. 8. Time dependence of the inactivation of lipoamide dehydrogenase by dioxan. Lipoate activity was measured as given in the methods. The enzyme, 0.16 mg/ml in 30 mM phosphate buffer pH 7.2 and 0.3 mM EDTA, in the dioxan concentrations indicated. ○—○, control; ●—●, 2%; ▲—▲, 6%; □—□, 10%; △—△, 20%. Temperature 25°.

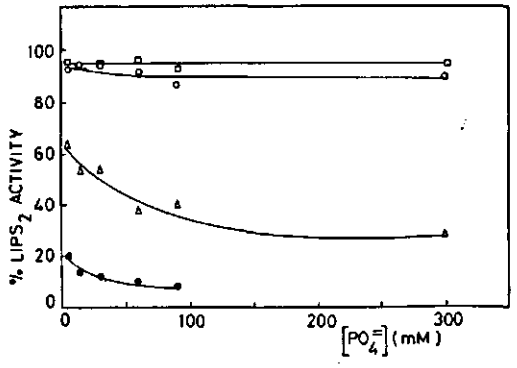


FIG. 9. Dependency of the inactivation of lipoamide dehydrogenase on the phosphate concentration. Lipoate activity was measured as given in the methods. Enzyme, 0.13 mg/ml in 30 mM phosphate buffer pH 7.2 containing 0.3 mM EDTA, with and without 20% dioxan at the times indicated. □—□, control, measured directly; ○—○, control, after 4h incubation; △—△, with 20% dioxan, measured directly; ●—●, with 20% dioxan, after 4 h incubation. The enzyme solutions were incubated on ice.

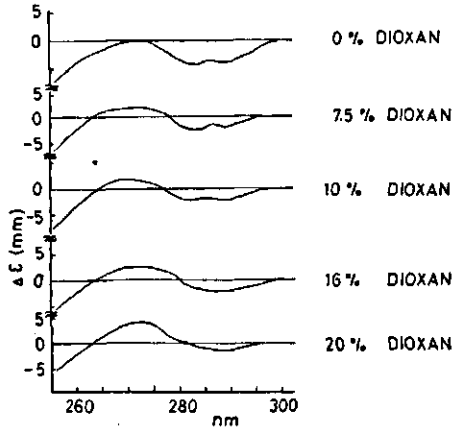


FIG. 10. Dependence of the CD spectra of lipoamide dehydrogenase on the dioxan concentration. Enzyme: 1.34 mg/ml in 30 mM phosphate buffer pH 7.2 containing 0.3 mMEDTA, in the dioxan concentrations indicated; A, control; B, 7.5%; C, 10%; D, 16%; E, 20%. Temperature 25°.

rate of inactivation (Fig. 9). In the presence of ammonium sulfate the enzyme denatures and precipitates. It is possible that part of the inactivation of enzyme upon incubation with dioxan or 2-chloroethanol is reversed upon dilution in the activity mixture. A control experiment in which 20% concentration of the pertuband was incubated in the assay mixture shows the same initial activities as in the absence of these solvents in the assay mixture.

The CD spectrum of the holoenzyme shows in the 250–300 nm region at least two negative bands. These bands disappear upon dissociation and a positive

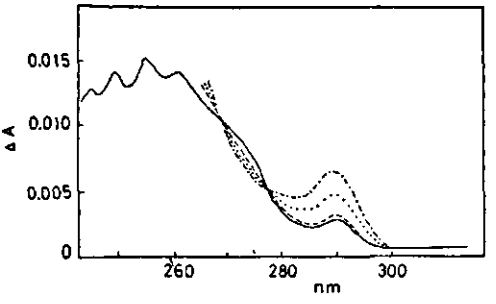


FIG. 11. Difference spectra of enzyme, 1.6 mg/ml, and enzyme, 1.6 mg/ml in 30 mM phosphate buffer pH 7.2 and 0.3 mM EDTA, with dioxan; ——, 7.5%; — — —, 10%; . . .., 12.5%; -.-.-, 16%. Temperature 25°.

band around 270 nm appears (chapter 4). Upon the incubation of the enzyme with dioxan and 2-chloroethanol the two negative bands at 280–290 nm decline and a weak positive band at 270 nm is observed. The magnitude of these effects is dependent on the time of incubation and on the concentration of the pertubants (Fig. 10).

Difference spectra of lipoamide dehydrogenase with several dioxan concentrations were made. In the 260–300 nm region a difference in absorbancy is observed (Fig. 11). Since the spectrum of FAD in this wavelength region is strongly dependent on the solvent and more sensitive than the bands in the visible region (MILES and URRY, 1968) it is likely that the difference spectrum is not solely due to pertubations around tryptophan and tyrosine. The difference spectrum has a peak at about 290 nm, suggesting that a tryptophan becomes exposed to the medium. At lower wavelengths a broad peak is observed which could consist of FAD as well as tyrosine and phenylalanine contributions.

As dissociation of the enzyme upon dilution is accompanied by a change in environment of a tryptophan residue (chapter 4) it was of interest to see whether addition of dioxan causes dissociation. On a Sephadex G-200 column, eluted with 30 mM phosphate buffer pH 7.2 and 0.3 mM EDTA with 20% dioxan, two protein peaks are observed. The molecular weight of these peaks cannot be calculated in the normal way, due to the changed swelling properties of Sephadex G-200 in dioxan, which causes the peaks to be eluted at volumes too large for corresponding with their molecular weight (DETERMANN, 1967). But still the species with the highest molecular weight will be eluted first. Therefore it seems not unreasonably to assume that dioxan has shifted the monomer-dimer equilibrium towards the monomer side since both peaks contained protein and the second peak was not observed in a control experiment without dioxan. Both peaks contained flavin but were inactive.

3.3. DISCUSSION

The fact that lipoamide dehydrogenase has to be dialysed extensively against NaCl solutions in order to obtain a spontaneously dissociating enzyme suggests that there is an exchange of ions. If the dialysis is carried out for a not too long time less dissociation is observed, indicating a mixture of dissociating and non-dissociating enzyme. Together with the fact that stabilization occurs in the presence of high phosphate concentrations, in our experiments always the sodium salt, these results suggest that protein-bound phosphate groups are exchanged for one or more chloride ions. In addition the observation that halogen ions interfere with the recombination of the apoenzyme and FAD (VISSER, 1970) indicates the prevention of interaction between phosphate and the enzyme. Furthermore it is necessary to dissolve the apoenzyme first in 0.2 M phosphate buffer pH 8.2 before effective binding of FAD and association of the apoenzyme-FAD monomer occurs (VISSER, 1970).

When we relate the percentage lipoate activity with the percentage dimer

present, as was shown to be correct for the dissociation upon dilution (VISSER, 1970), it is surprising that the highest percentage of monomer is found at the lowest NaCl molarity. This suggests that the dissociation is not related to the binding of NaCl molecules but to the removal of phosphate ions. This shows that the reason for dissociation of lipoamide dehydrogenase is quite different from the process of dissociation by neutral salt molecules as is observed for a number of proteins (FRIDOVIC, 1963, SWANEY and KLOTZ, 1970, CONSTANTINIDES and DEAL, 1970, KAWAHARA et al., 1965, BENESCH et al., 1964 and GAWRONSKI and WESTHEAD, 1969). In the latter cases a Hofmeister serie is found for the effectiveness of the different salts while in our case the result is rather independent on the type of salt.

Dissociation of the enzyme can also be achieved by freezing the enzyme and by preparing the apoenzyme (VISSER, 1970). In case of the apoenzyme the tryptophyl residues are in a more polar environment than in the holoenzyme. Tryptophyl residues become also more exposed to the medium by treating the enzyme with dioxan and chloroethanol. The α-helix content does not change in dioxan and 2-chloroethanol, while the α-helix content decreases upon dissociation (chapter 4). These organic solvents are helix-promoting, due to a shift of apparent pK values of acidic groups to higher pH (FINDLAY, 1962, JIRGENSON, 1969). These two opposite effects may result in a not observable change in the ORD measurements. Thus depending on the composition of the medium the monomer has different conformations. Papaïn shows no change in ORD spectrum up to 70% methanol. The loss of activity is ascribed in the latter enzyme to the interference with hydrophobic interactions between enzyme and substrate (SLUYTERMAN, 1967). Apart from this helix-promoting effect it is also believed that dioxan causes contraction of proteins (IIZUKA and YANG, 1965). The proteins contract in order to minimize the exposure of polar side chains to the, because of dioxan, more hydrophobic medium. At high dioxan and 2-chloroethanol concentrations the proteins will precipitate with aggregation as is observed in the case of lipoamide dehydrogenase. In this respect it is of interest that increased phosphate concentrations promote the dissociation in the presence of dioxan, rather than inhibit the dissociation as in the absence of dioxan. Since high concentrations of phosphate promote the hydrophobic interactions (VISSER, 1970), it is likely that the presence of dioxan interferes with the formation of the proper conformation, thus leading to increased dissociation and inactivation.

The precipitation at low ionic strength is possibly due to a decrease in the activity coefficient of the protein, the reverse process is known as salting in. The influence of salts on proteins seems to be related to the hydratation of proteins (LUMRY and BILTONEN, 1969). However when under the circumstances where effects are observed the amount of salt ions is small compared with the amount of water present, changes in hydratation may, according to views in the literature (TANFORD, 1969), be ignored. On the other hand the requirement to remove phosphate effectively from the interior of the protein does, in our opinion, not exclude the possibility of changes in hydratation of the protein.

4. PROPERTIES OF THE DIMERIC ENZYME, THE MONOMERIC ENZYME AND THE APOENZYME

4.1. INTRODUCTION

It was shown in the preceding chapter that a monomer-dimer equilibrium exists under various conditions. It was of interest to study the ORD, CD, and fluorescence properties of the monomer and the dimer. In this chapter the properties of the dimer, the monomer obtained upon dilution and the apoenzyme are compared.

It has been reported by SIMPSON and VALLEE (1966) that lipoamide dehydrogenase has, due to the flavin chromophoric group, an optical active absorption band at about 370 nm, while the 260 nm band is probably concealed with the peptide backbone rotation. Changes in the ORD behaviour in the 200–300 nm region, believed to be due to side-chain interactions, are difficult to interpret for proteins containing chromophoric groups (YANG, 1967; CARVER et al., 1966; FASMAN et al., 1964; TIMASHEFF et al., 1966; COLEMAN and BLOUT, 1967). The lipoamide dehydrogenase holoenzyme (dimer) contains per peptide chain one FAD prosthetic group, two tryptophyl, seven tyrosyl residues, eight -SH groups and one intrapeptide S-S bridge.

Small Cotton effects can be amplified by the use of Moffitt-Yang plots (TIMASHEFF et al., 1966). In this way it is sometimes possible to identify the group responsible for the optical activity, on the other hand in case of several Cotton effects within a small wavelength region shifts in the wavelength of the Cotton effects due to overlapping may occur which again makes identification of the group involved uncertain (TIMASHEFF et al., 1966; COLEMAN and BLOUT, 1967). Sometimes it is possible to get more information from CD spectra as the dichroic bands are finite and have a gaussian shape. However also here it is very difficult to assign certain effects to changes of a particular amino acid. The total CD spectrum has to be resolved in a set of gaussian curves, with the bands correlated to the absorption spectrum as good as possible. A few studies have been made in this way with flavoproteins (D'ANNA and TOLLIN, 1970; EDMUNDSON and TOLLIN, 1970). In general however it is not possible to draw conclusions from CD spectra alone in complex absorption regions, but it has been possible to correlate the circular dichroism spectra with the interactions which occur at the coenzyme binding site of flavoproteins (D'ANNA and TOLLIN, 1970; EDMUNDSON and TOLLIN, 1970).

The behaviour of FAD, tryptophan and tyrosine can also be followed by fluorescence (MASSEY, 1963; RABINOWITCH, 1968; WEBER and YOUNG, 1964). Since lipoamide dehydrogenase, in contrast to other flavoproteins, shows a strongly enhanced flavin fluorescence (MASSEY, 1963), it was of interest to study this property upon dissociation in relation with the protein fluorescence. A combination of the different methods used thus provides information to identify the

nature of the observed changes in ORD and CD parameters.

Transfer of excitation energy between groups of a protein is under certain circumstances a known effect (FÖRSTER, 1947). Back in 1963 (MASSEY, 1963) energy transfer from the protein chromophores to the FAD chromophore in lipoamide dehydrogenase was postulated.

4.2. RESULTS

4.2.1. *ORD studies*

Optical rotatory dispersion curves at several concentrations of enzyme were made at 7°. In agreement with SIMPSON and VALLEE (1966) a Cotton effect attributable to the FAD-prosthetic group could be detected at 350 nm. This interpretation is in agreement with the observation, which will be shown later in this chapter, that the CD spectrum of lipoamide dehydrogenase shows a positive band with at least two maxima at 360–370 nm, which is identical with the near-UV absorption band.

At concentrations where the dimer exist, in this case higher than 0.3 mg/ml, the negative peptide rotation shows a minimum at 233 nm, $[m'] = 5{,}000$, with a cross-over point at 224 nm (Fig. 12). At concentrations less than 0.1 mg/ml the 350 nm FAD band can still be detected but the minimum of the peptide rotation has shifted considerably, from 233 nm to 238 nm, $[m']_{233} = 1{,}800$ and $[m']_{238} = 3{,}800$. Furthermore a decrease in its magnitude is observed while the cross-over point shifts from 224 nm to 230 nm.

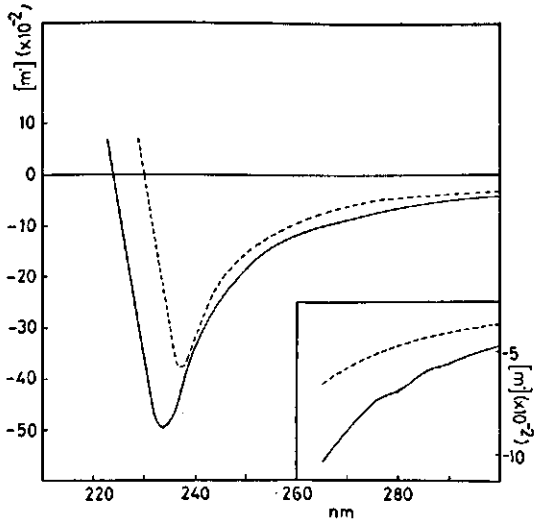


FIG. 12. The influence of the protein concentration upon the ORD curves of lipoamide dehydrogenase. The experiment was carried out in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA. Protein concentration: ——, 0.12 mg/ml; – – –, 0.50 mg/ml. Temperature 7°.

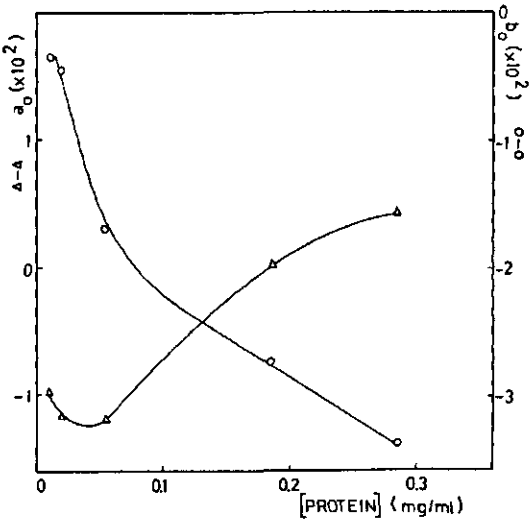


FIG. 13. Dependency of the Moffitt-Yang parameters a_0 and b_0 on the protein concentration. △—△, a_0; ○—○, b_0. The experiment was carried out under the conditions described in Fig. 12.

The ORD spectra also show Cotton effects in the 260–300 nm region, but only at concentrations were the dimer exists. Moffitt-Yang plots for several enzyme concentrations show pertubations in the 260–300 nm region; these pertubations cannot be eliminated by substituting other λ_0 values as suggested by

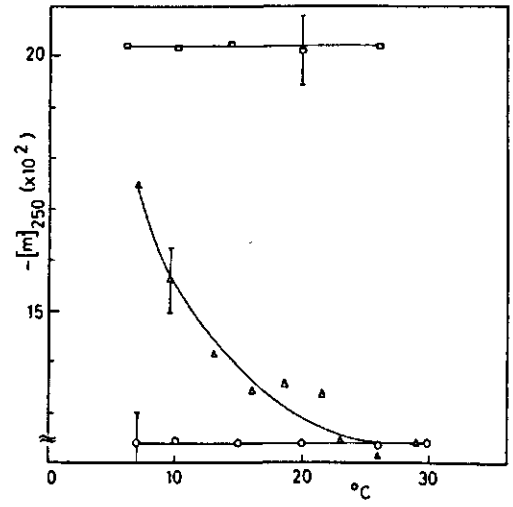


FIG. 14. Dependency of the optical rotation of lipoamide dehydrogenase on the temperature. The results are expressed as molecular rotation at 250 nm. Protein concentration: □—□, 0.50 mg/ml; △—△, 0.13 mg/ml; ○—○, 0.08 mg/ml in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA.

FASMAN (1963). Therefore they represent real Cotton effects. Plots of the Moffitt-Yang parameters a_0 and b_0 against the enzyme concentration are shown in Fig. 13; a_0 and b_0 are concentration-dependent below 0.3 mg/ml. Furthermore a drastic change in the slope of both plots is observed at about 0.05 mg/ml At about 0.1 mg/ml the red shift of the cross-over point and the minimum of the ORD-spectrum occurs.

The ORD curves are also dependent on the temperature, at a concentration between 0.3 mg/ml and 0.1 mg/ml, as is shown in Fig. 14 where $[m']_{250}$ is plotted against the temperature. Similar relationships are found for [m'] at other wavelengths in the 245–280 nm region.

At a concentration less than 0.1 mg/ml a gradual change in the ORD parameters with the time is observed. Fig. 15 shows these changes upon incubation on ice. After an initial stable period b_0 declines for several hours after which an increase is found. The b_0 value found after 50 hours is approximately double its initial value.

As will be shown in Fig. 16 the apoenzyme has a higher apparent α-helix content than the holoenzyme in its dimeric state. It is likely that the change in b_0 at this low enzyme concentration reflects the formation of the apoenzyme since $K_{ass.} = 3.3 \times 10^5\ M^{-1}$ for FAD with the monomeric apoenzyme at 10° (VISSER, 1970). In agreement with this, the opposite effect is found when the ORD changes occurring upon recombination of FAD with the apoenzyme are followed with time. Because of the high absorbancy of the FAD solution used, the experiment was carried out at 240 nm. At this wavelength, under the conditions used, the

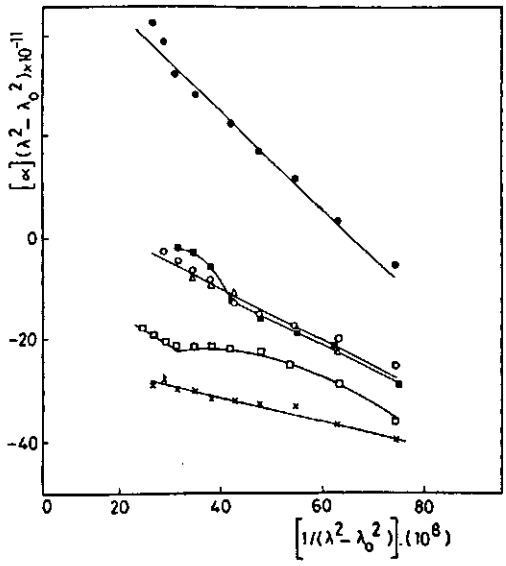


FIG. 15. Influence of time on the Moffitt-Yang plots of lipoamide dehydrogenase. Protein concentration 0.03 mg/ml in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA. ●—●, immediately after dilution; △—△, after 2 h; □—□, after 4 h; ×—×, after 6 h; ■—■, after 8 h; ○—○ after 50 h. Temperature 25 °C.

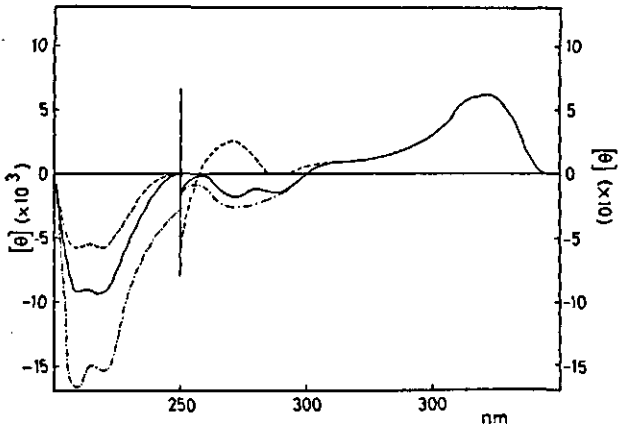


FIG. 16. CD spectrum of lipoamide dehydrogenase and its apoenzyme. Enzyme concentration: ——, 0.5 mg/ml; - - - -, 0.10 mg/ml; -.-.- apoenzyme. The experiment was carried out at 5° in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA.

correction of [α] for the decrease in free FAD, due to binding, can be ignored because of the 10-fold excess of flavin.

TABLE 1. The effect of FAD binding on the ORD parameters of lipoamide dehydrogenase apoenzyme.

		Apo + FAD			Apo + FAD + FMN			Apo + FAD + FMN		
	Time h.min.	lip-S_2 (%)	DCIP (%)	-[m] ($\times 10^{-2}$)	lip-S_2 (%)	DCIP (%)	-[m] ($\times 10^{-2}$)	lip-S_2 (%)	DCIP (%)	-[m] ($\times 10^{-2}$)
	10	30	–	44						
	20	40	850							
	30	–	1300							
	40	57	1200							
	50	–	900							
1		60	550	44	60	500	43			
1	20	–	–		51	–	–			
1	30	60	–	45	48	–	44			
2	20	65	570		–	390	–	66	390	
3		65	–	40	48	360	–	68	360	
24		–	–	40	–	–	40	–	–	42
25		72	400	40	46	390	40	49	390	40
48		76	300	30	39	320	33	45	300	40

Holoenzyme: lipS_2 activity, 41; DCIP activity, 280. No inactivation of the enzyme was observed upon storage under the conditions of the experiment; specific rotation, $[m']_{240}$: – 3600. Apoenzyme: lipS_2 activity, 2.5; DCIP activity, 360; specific rotation, $[m']_{240}$: – 3700. Activities are expressed relative to those of the holoenzyme. Calculations are based on $A/\frac{1cm}{1mg/ml} = 1.0$ for both apoenzyme and holoenzyme. 0.01 ml 10 mM FAD was added to 3 ml apoenzyme (0.9 mg/ml) in 30 mM phosphate, pH 7.2, plus 0.1 mM EDTA (20°); at the times indicated 0.005 ml 100 mM FMN was added to 0.5 ml apoenzyme-FAD mixture. Dilutions due to the addition of FAD and FMN are neglected.

From Table 1 it can be seen that 10 minutes after the addition of FAD to the apoenzyme the specific rotation has increased over that of the apoenzyme. During the time the lipoate-reductase activity increases – e.g. during the association process (VISSER, 1970) – little change in [α] occurs. It is surprising that after this activity has reached its highest value [m'] is still much higher than the corresponding values of the holoenzyme and of the apoenzyme. Furthermore [m'] of the monomer (Fig. 12) is lower than that of the dimer at this wavelength. When about 60% of the lipoate activity has returned, e.g. dimeric enzyme is formed, the 2,6-dichlorophenolindophenol (DCIP) activity is still six times higher than that of the holoenzyme. During prolonged incubation at 25° the DCIP activity declines gradually without any change in the lipoate-reductase activity. During this time [m'] also declines and approaches the value of the holoenzyme. It can be argued that not all the apoenzyme is converted into the dimeric enzyme as judged from the specific activity of the reactivated enzyme (75% of the original activity). The decline in [m'] and DCIP activity thus can be due to loss of DCIP active enzyme by denaturation upon prolonged standing at 25° (VISSER and VEEGER, 1970). It cannot be excluded however that we are dealing with a fully reactivated dissociating enzyme (chapter 3) since it has been shown by VISSER (1970) that a long time is needed before the FMN sensitivity has disappeared. As expected from previous studies (VISSER, 1970) FMN inactivates both the DCIP activity and the lipoate activity of the reactivated enzyme to about the same extent and keeps the [m'] at a higher value.

4.2.2. *CD studies*

The CD spectrum of lipoamide dehydrogenase (Fig. 16) shows several optical active absorption bands when recorded at concentrations where the dimer exists, in agreement with the ORD data. Our results are only in partial agreement with those shown by BRADY and BEYCHOK (1969). In the 350–400 nm region a small and broad positive dichroic band, belonging to the FAD chromophore, is found. At least two bands are visible in this region. Compared with the CD spectra of MILES and URRY (1968) and in agreement with the increase in fluorescence intensity of the bound FAD (MASSEY, 1963), the enzyme-bound flavin seems to have a FMN-like conformation. On the other hand the negative band of FMN (MILES and URRY, 1968) above 400 nm cannot be detected. The red shift of the maximum of the 360 nm band compared with the 340 nm band of free FMN leads to a good agreement with the absorption spectrum. The molar ellipticity [θ] for FAD in the 360 nm band is about 0.5% of the value reported by MILES and URRY (1968) for free FMN, however our value is based on the mean residue weight of the protein. Based on the flavin content of the protein and the molecular weight of the flavin the molar ellipticity of the 360 nm band becomes about 450 times higher and is about 2.5 times higher than that of free FAD, indicating an increased dichroism due to binding to the protein. An almost similar value of [θ] for holoenzyme-bound flavin has been reported by BRADY and BEYCHOK (1969).

The peptide CD bands dominate over the UV bands of the FAD. In the 280–

290 nm region the CD spectrum of the dimer shows two small negative bands, while two negative bands at 218 nm and 208 nm, characteristic for the α-helix, are observed. Upon dilution, thus upon dissociation, the two small minima at 280 nm and 290 nm disappear, while at 270 nm a positive band of the same magnitude appears. Furthermore the α-helix bands decrease upon dilution, but the ratio $[\theta]_{218}/[\theta]_{208}$ remains constant.

The CD spectrum of the apoenzyme was also recorded in the 200–300 nm region. The magnitude of the maxima at 208 and 218 nm and at 270–280 nm is much higher than those of the holoenzyme; the latter maxima have shifted towards the blue in the apoenzyme. This result suggests a higher α-helix content of the apoenzyme. In contrast to that of the holoenzyme the CD spectrum of the apoenzyme is not concentration-dependent. Our result with the apoenzyme differs completely from that of BRADY and BEYCHOK (1969). The spectrum found by these authors may differ from ours because of the possible presence of denatured protein in their preparation, caused by guanidine used in their preparation method.

4.2.3. *Fluorescence studies*

Since the ORD and CD data suggest a conformational transition upon dissociation around one of the side-chain chromophores of the enzyme which has dichroic bands in the 250–300 nm region, it was important to study under these conditions the changes in fluorescence of both the protein and the bound flavin. This is of special interest since DE KOK et al. (1968) have concluded from lifetime studies that although the isoalloxazine-adenine complex is broken in the holoenzyme quenching due to a group other than adenine, probably a -SH group in the vicinity of the flavin, occurs.

The emission spectra of lipoamide dehydrogenase, excitation at 290 nm, are concentration-dependent. The ratio of the protein fluorescence emission at 330 nm and the flavin emission at 520 nm starts to decline below a protein concentration of 0.2 mg/ml and parallels the concomitant inactivation of the enzyme (Fig. 17A). This decline in ratio is due to an increase of the protein fluorescence upon lowering of the protein concentration, whereas only a slight increase in quantum yield of FAD is observed. In order to investigate whether the increase in protein fluorescence is due to a decrease in energy transfer the flavin emission at 520 nm, obtained by excitation at 360 nm, was normalized for the concentrations used by signal amplification to the same recorder deflection and the total excitation spectrum registrated at different temperatures. The excitation maxima of the flavin emission found at 450 nm and 360 nm are rather independent of the protein concentration at all temperatures measured. Between 260 nm and 290 nm large temperature-dependent differences in the excitation spectrum of the flavin emission occur. The ratio of the excitation maxima (Ex_{360}/Ex_{290}), corrected for the same number of excitation quanta, declines upon dissociation (Fig. 17B). The ratio is initially not very temperature dependent. Above 11° however it declines, goes through a minimum at 15° and then starts to increase again to a final value which is higher than the initial one. Although

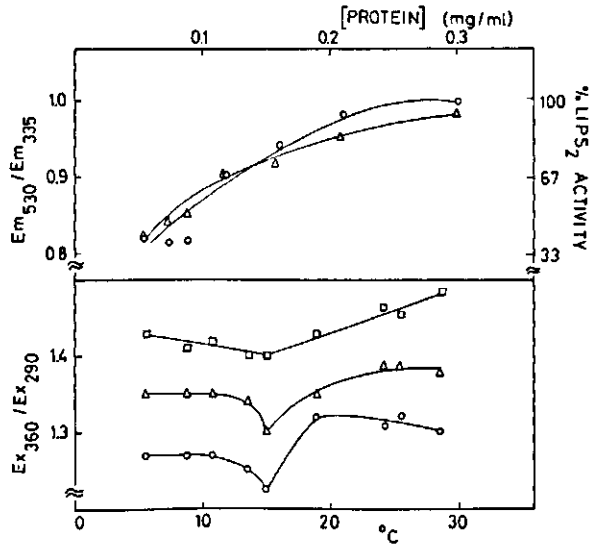


FIG. 17A. Relation between fluorescence emission of protein (E_{335}) and flavin (E_{520}) and the protein concentration. The ratio of flavin emission at 520 nm and protein emission at 355 nm obtained by excitation at 290 nm (△—△) is compared with the lipoate activity (○—○) at different concentrations. B; Dependence of the flavin fluorescence excitation ratio, Ex_{360}/Ex_{290}, on the temperature. Emission wavelength: 515–545 nm. Protein concentration: □—□; 0.30 mg/ml, △—△; 0.15 mg/ml, ○—○; 0.08 mg/ml in 30 mM phosphate buffer pH 7.2 containing 0.3 mM EDTA.

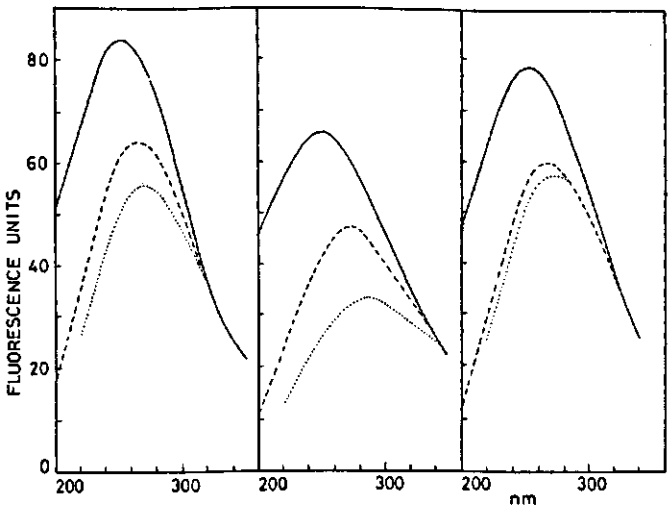


FIG. 18. Protein fluorescence emission spectra of lipoamide dehydrogenase. Excitation wavelength: ———; 280 nm, – – – –; 292 nm, ...; 297 nm. Protein concentration: A; 0.3 mg/ml at 25°, B; 0.1 mg/ml at 5° and 25°, C; 0.3 mg/ml at 5°, in 30 mM phosphate buffer pH 7.2 containing 0.3 mM EDTA. Spectra not corrected for photomultiplier sensitivity, were normalized to the same recorder deflection at 375 nm.

there are quantitative differences between the results obtained a similar effect of the temperature is observed for both the dimer and the monomer, indicating that this process is rather independent on the state of aggregation.

Protein emission difference spectra were made at different concentrations of protein and at varying temperatures according to the method of WEBER and YOUNG (1964). At the protein concentrations given in Fig. 18 an emission maximum at 325–326 nm is observed upon excitation at 280 nm. The difference between the emission spectra of the protein solutions excited at 280 nm and at 292 nm shows that the temperature has little influence on the relative amount of tyrosine fluorescence; on the other hand the quantum yield increases upon dissociation.

Both the temperature and the protein concentration affect the nature of the tryptophan emission. At 5°, at concentrations where the dimer exists, there is hardly any difference between the emission spectra excited at 292 nm and at 297 nm, while at 25° a slight difference is observed. Larger differences between the maxima of the emission bands (335 nm and 345 nm), obtained by excitation at respectively 292 nm and 297 nm, are observed upon dissociation.

4.3. DISCUSSION

Upon dilution lipoamide dehydrogenase dissociates with concomitant changes of certain parameters. Changes of the ORD parameters upon dissociation have been observed with other enzymes (MCKENZIE et al., 1967; SWAISGOOD and TIMASHEFF, 1968), but not always a decrease in apparent α-helix content is detected upon dissociation. The changes occurring in this enzyme upon dissociation seem to indicate a decrease in α-helix content from 30% to 20%. This conclusion seems not unreasonable since the values obtained by CD measurements and ORD measurements are in rather good agreement. On the other hand the shift of the ORD minimum is difficult to explain. A change from α-helix to random coil or β-helix is expected to give a blue shift of the minimum (GREENFIELD et al., 1967) and the ratio $[\theta]_{218}/[\theta]_{208}$ in the CD spectrum to change. Another possibility is a drastic change around one or more of the chromophores or side-chains. The possibility that FAD is the group involved and that the flavin in a FMN-like conformation has optical active bands in this region cannot be ruled out. This may be true despite the observation that within experimental error no changes in the 300–370 nm region are detectable, since these latter bands are rather insensitive to pertubations (MILES and URRY, 1968). On the other hand changes in particle size and shape and Duysens scattering can also cause these kind of changes (URRY and JI, 1968). A change in Duysens scattering would imply aggregation of the inactive monomer. In fact aggregation has been observed with the dissociating enzyme at low ionic strength (chapter 3). In this respect the enzyme resembles glutamate dehydrogenase, which also aggregates a-specifically after initial dissociation into 50.000 molecular weight monomers (EISENKRAFT, 1969).

It is surprising that the monomeric apoenzyme has twice the α-helix content of the holoenzyme. On the other hand the method of preparing the apoenzyme c.f. in the presence of 1 M KBr, could be the reason for this difference. There is a rather good agreement between the values calculated from CD and ORD measurements. An equally high α-helix content in the apoenzyme was not found by BRADY and BEYCHOK (1969); the much lower value found can be due to the method used to prepare the apoenzyme e.g. by dialysis against guanidine-HCl, which is an unfolding agent. Other apoenzymes are also known to have a lower α-helix content than the holoenzyme, for instance glucose oxidase (COSANI et al., 1968). The fact that BRADY and BEYCHOK (1969) found fully reactivated enzyme after recombination with FAD needs not to be in contradiction with our results as the recombination experiment shows that minor conformational changes occur a long time after maximum lipoate activity has been obtained. Also the fact that FMN inactivates the reactivated enzyme (VISSER, 1970), while it has no influence on the holoenzyme, shows that the lipoate activity can be obtained with a conformation of the whole enzyme which differs from that of the native one, presumably the dissociating enzyme.

Several amino acids and flavins have optical active bands in the 240–300 nm region (BEYCHOK, 1967). Reversal of the sign of a CD band in the 240–300 nm region has been observed in other studies (MILES and URRY, 1968) and are explained in terms of changes in solvent, pH or temperature. Only in a few cases (COSANI et al., 1968) it was possible to correlate these changes in CD bands with the pertubation or ionization of certain groups. According to TIMASHEFF (TIMASHEFF et al., 1967) the small negative CD minima at 280–290 nm which are found in films of poly-L-tryptophan could be attributed to a non-random arrangement of the tryptophyl residues. The origin of the small positive CD band at 270 nm which replaces the 280–290 nm bands upon dissociation cannot be attributed to a certain group or side chain. An attractive possibility is a pertubation around the FAD – which has dichroic bands in this region – because there is a close interaction between FAD and a tryptophan (VISSER, 1970); for instance MCCORMICK (1970) has suggested the possible importance of tryptophan in flavin binding in the enzyme flavodoxin.

At higher wavelengths, 300–500 nm, the CD spectra of the monomer and the dimer are identical. In the 360 nm region a small broad positive band belonging to the FAD chromophore is found. Free FAD has little or no optical activity above 400 nm, because of the interaction between the adenine moiety and the isoalloxazine nucleus. However FMN has optical activity in this region. This optical activity increases when FMN is bound to protein (D'ANNA and TOLLIN, 1970).

The fluorescence data indicate that interactions exist between FAD and the protein. The results show that the decrease in ratio of the protein emission to the flavin emission is due to an increase in protein fluorescence emission. This conclusion is supported by the protein excitation data since the difference spectra in Fig. 18 show that upon dissociation considerable unfolding occurs. At least one of the tryptophyl residues is pertubed to a slightly more polar environment, this

conclusion is confirmed by the CD data. The identical effect of the temperature on the Ex_{360}/Ex_{290} ratio of the dimer and the monomer indicates that only minor structural changes in the subunits around the FAD occur upon dissociation. The energy transfer in the monomer is more effective than in the dimer, probably because of a less effective radiationless drain of energy, which might be due to the different tryptophyl arrangement in the monomer, or to an increased overlap of the spectral bands caused by the slight environmental pertubations occurring upon dissociation (VISSER, 1970). The best explanation of the Ex_{360}/Ex_{290}-temperature pattern observed is that, because of slight local pertubations under the influence of temperature, distance and orientation of the excitation center and flavin vary. But it cannot be excluded that structural changes of water are responsible for this effect (DROST-HANSEN, 1967).

5. BINDING OF SODIUM DODECYLSULFATE TO LIPOAMIDE DEHYDROGENASE

5.1. INTRODUCTION

The interaction between proteins and sodium dodecylsulfate has been studied by several authors (RAY et al., 1966, REYNOLDS et al., 1967, REYNOLDS and TANFORD, 1970a, REYNOLDS and TANFORD, 1970b, FISH et al., 1970, REYNOLDS et al., 1970, REYNOLDS and HUANG, 1969, SMITH-JOHANNSEN and DRYSDALE, 1969, WEBER and OSBORN, 1969). Originally such studies were undertaken to obtain a model of the molecular cell architecture or to investigate the nature of the weak interactions between protein- and lipid components of cellular membranes. In the case of membrane structures the importance of lipophilic associations between lipids and proteins are of more importance than charge-charge interactions. Nevertheless charge-charge interactions are clearly demonstrated between lipids and membrane proteins (BRAUN and RADIN, 1969). Myelin can bind large amounts of both anionic and nonionic lipids. The binding of the two different lipids is independent and the results show that both binding regions are accessible at the same time. Abolishment of the lipid binding capacity of myelin after succinylation of the ε-amino groups of its lysine is observed, possibly due to changes in the structure of the protein induced by the introduction of the negative charge (BRAUN and RADIN, 1969). A change in configuration of a protein may destroy the availability of the binding sites but new sites may become available at the same time.

Effects of binding of lipids to proteins are ascribed in terms of alteration of binding forces or conformational changes. The conformational changes can be expressed in exposure of groups to a different environment upon lipid binding. In case of RNA-se, which undergoes a structural transition in 0.15 sodium dodecylsulfate, the histidyl groups become exposed (STARK et al., 1961). Except histidyl also tyrosyl groups are claimed to become exposed to the medium in case of RNA-se (COWGILL, 1964, PITTZ and BELLO, 1971).

Exposure of tyrosine residues is in RNA-se only observed with an anionic detergent but with other proteins, for instance insuline (SHAPIRO et al., 1967), interactions with non-ionic detergents cause exposure of tyrosine residues to the medium. In rabbit phosphoglucose-isomerase buried sulfhydryl groups become exposed to the medium (COWGILL, 1964).

The dependence of the interaction of protein with ionic detergents on the pH and ionic strength is not always found (COWGILL, 1964). Modelstudies show that binding of detergents to small molecules is highly dependent on the charge of the molecule, while binding to proteins is regarded as highly hydrophobic (PITTZ and BELLO, 1971).

In many cases protein molecules are built up from subunits, these subunits are held together by hydrophobic forces. In these instances SDS binding can

compete with subunit interaction and will cause dissociation at high SDS concentration. A number of examples are described (SHIREY and HUANG, 1969, WEBER and OSBORN, 1969, SHAPIRO et al., 1967, COWGILL, 1964, COWGILL, 1966, COHN and MCMANUS, 1971). In order to explain the peculiar effect of protein concentration in SDS binding studies RAY et al. (1966) suggested the opposite effect, e.g. dimerization upon SDS binding also occurs by assuming that a long-chain alifatic molecule can adhere to more than one protein molecule. Another possibility, competition with residually bound fatty acid, should not lead to dimerization.

REYNOLDS and TANFORD (1970b) found that proteins, which do not contain disulfide bridges, appear in SDS to be rods of radius and length proportional to their molecular weights, this in contrast to the same proteins in the absence of SDS. According to COWGILL (1966) SDS cannot break disulfide bridges, when the protein contains one or more disulfide bridges the elution pattern, in gel chromatography, of the protein-SDS complex is different from that of the reduced proteins-SDS complex and the relation between length and molecular weight does not longer exist.

Reduced protein binds 1 g SDS/1 g protein at low monomer-detergent concentration, while at high SDS concentration 1.4 g SDS/1 g protein is bound. Effects observed upon SDS treatment can also be observed with other pertubants. For instance unreduced BSA in guanidine-HCl has the same hydrodynamic properties as unreduced BSA with SDS (FISH et al., 1970), while the ultraviolet difference spectrum of BSA in the presence of SDS is the same as the ultra-violet difference spectrum of BSA in the presence of sucrose or of ethylene glycol (RAY et al., 1966).

Studies in our laboratory showed that lipoamide dehydrogenase is a dimer with no S-S bridges between the subunits, the S-S bridges are all intramolecular (KALSE and VEEGER, 1968). This dimer is able to dissociate at low pH and low ionic strength upon dilution (chapter 3). As the nature of the association process from apoenzyme to holoenzyme is of hydrophobic character (KALSE and VEEGER, 1968), it was of interest to study the interaction between sodium dodecylsulfate and the lipoamide dehydrogenase holoenzyme.

5.2. RESULTS

5.2.1. *Influence on the activity*

The influence of SDS on the activity of lipoamide dehydrogenase is shown in Fig. 19. The decrease in activity occurs at a certain SDS concentration which is dependent on the enzyme concentration, a higher SDS concentration is needed at increasing enzyme concentrations. The absence of any effect by SDS up to a certain concentration and its dependence on the enzyme concentration was observed with all methods used to study the influence of SDS on different parameters of the enzyme. In the SDS binding experiments, carried out at 20°, no formation of micelles can be observed in the presence of enzyme. In the absence

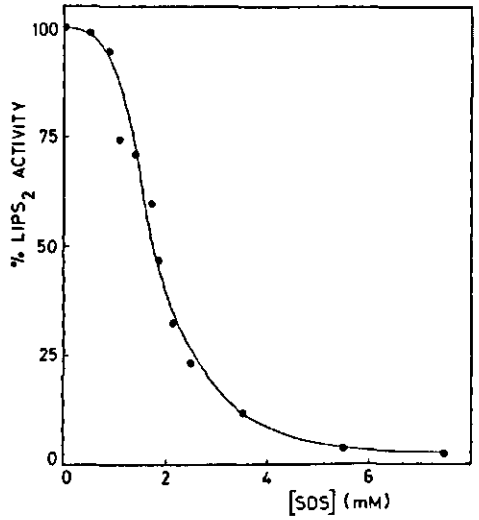


FIG. 19. Influence of SDS concentration on the activity of lipoamide dehydrogenase. To enzyme (1 mg/ml) in 30 mM phosphate buffer pH 7.2, SDS was added and the activity measured in the NADH-lipoate assay as given in the Methods. Temperature 25°

of enzyme the detergent easily forms micelles (REYNOLDS and TANFORD, 1970a). However at 0° with the same concentration of SDS formation of micelles easily occurs in the presence of enzyme. When a mixture of enzyme and SDS at 20° is additionally incubated for two hours on ice the SDS molecules form micelles and precipitate while the enzyme activity slowly returns (Fig. 20); the percentage of reactivation is dependent on the SDS concentration of the incubation mixture by raising the temperature to 20° the activity declines again and the micelles disappear.

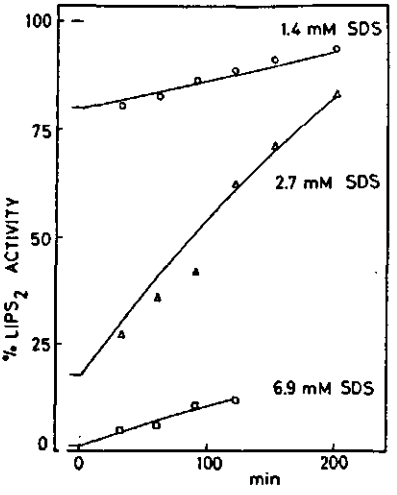


FIG. 20. Influence of the temperature on SDS-inactivated lipoamide dehydrogenase. Enzyme (1 mg/ml) in 30 mM phosphate buffer pH 7.2 was treated at 25° with SDS; ○—○, 1.4 mM SDS; △—△, 2.7 mM SDS; □—□, 6.9 mM SDS. After measuring the activity as in Fig. 1, the temperature of the sample was lowered to 0° and at different times the activity of an aliquot measured at 25°.

It was concluded by REYNOLDS and TANFORD (1970a) from SDS binding studies with a number of proteins that an important part of this binding is of hydrophobic nature. As SDS is an anionic detergent, Tween 80, a non-ionic detergent, was tested to distinguish between hydrophobic and ionic effects. Tween 80 does not have any influence on the activity of the enzyme.

5.2.2. *Influence on the spectral characteristics*

It is however possible to observe binding of Tween 80 to lipoamide dehydrogenase from difference spectra in the 300–600 nm region recorded of enzyme against enzyme plus different concentrations of SDS either in the presence or the absence of Tween 80. When SDS is added to an enzyme solution in the presence of Tween 80 at the early stages an increase in absorbancy in the near ultraviolet part of the spectrum (300–400 nm) is observed. This is due to the fact that enzyme-bound Tween 80 has a lower absorbancy than free Tween 80. The enzymically-bound Tween 80 is liberated upon the addition of SDS.

The spectra recorded in the 300–600 nm region in the presence of SDS, without Tween 80, show large spectral shifts, induced above a certain SDS concentration (Fig. 21A). The visible absorption spectrum bands shift towards shorter wavelengths since the difference spectrum shows a large negative maximum at 490 nm and a small negative maximum at 455 nm; furthermore positive differences at 390 nm and 440 nm are observed. The titration curves are bifasic. The spectral differences at 390 nm and 490 nm start to develop at a 50-fold molar excess of SDS over enzyme; on the other hand changes at 440 nm develop not below a 100 to 150-fold molar excess of SDS.

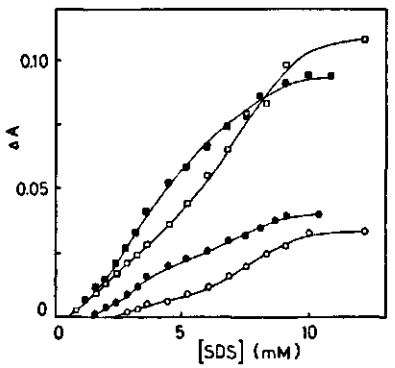
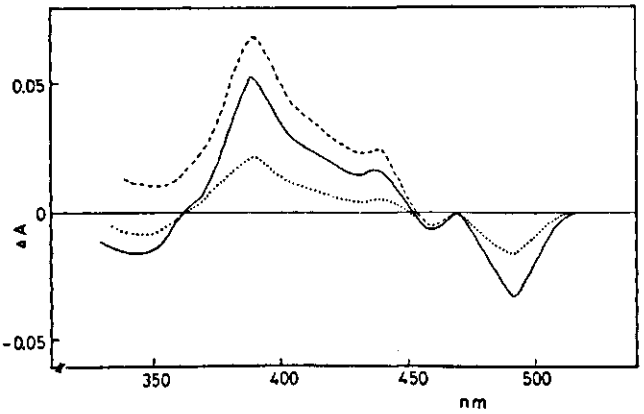


FIG. 21. Effect of SDS and Tween 80 on the spectrum of lipoamide dehydrogenase.
A: Enzyme (2 mg/ml), either in the absence or presence of a saturating concentration of NAD^+ (1.1 mM) in 30 mM phosphate buffer pH 7.2, was treated with different concentrations of SDS and the difference spectrum recorded against a blank of enzyme either without or with NAD^+ respectively. Temp. 20°.
○—○, absorbancy difference at 440 nm in the absence of NAD^+;
●—●, absorbancy difference at 440 nm in the presence of NAD^+;
□—□, absorbancy difference at 390 nm in the absence of NAD^+;
■— ■, absorbancy difference at 390 nm in the presence of NAD^+.



B: Enzyme (2 mg/ml) was titrated with SDS in the presence or absence of Tween 80. Difference spectra were recorded against a blank of enzyme. ———, difference spectrum at saturation with SDS (10 mM); . . . . ., difference spectrum with 3 mM SDS; – – – –, difference spectrum at SDS saturation (10 mM) in the presence of 1 mg/ml Tween 80,

When an enzyme solution was titrated spectrophotometrically with SDS, in the presence of Tween 80, a small positive absorbancy was observed in the 300–450 nm region. At a certain concentration of SDS the typical spectral changes as observed with SDS alone became visible (Fig. 21 B); the concentration of SDS needed to induce these changes is higher as compared with SDS alone. Further-

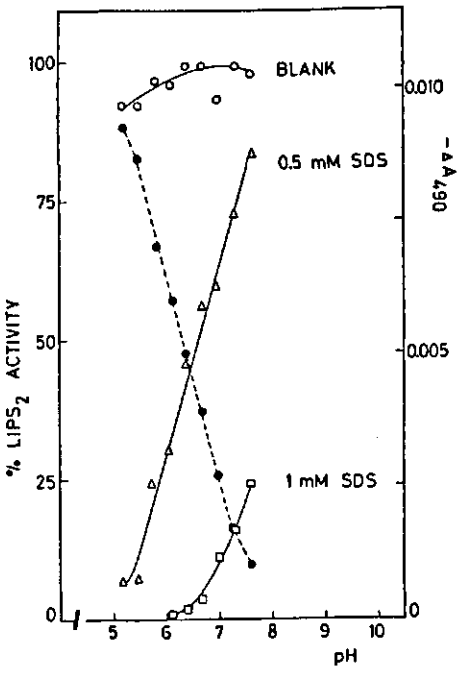


FIG. 22. The influence of the pH on the effect of SDS on the activity and the spectral properties of lipoamide dehydrogenase. To enzyme (0.1 mg/ml) in phosphate buffer (I = 0.1) at the pH given, SDS was added in the concentration: ○—○; no SDS △—△; 0.5 mM SDS, □—□; 1 mM SDS. The enzyme activity (solid lines) was measured as in Fig. 1. In the spectral experiment enzyme (0.3 mg/ml) was mixed with SDS (0.5 mM) and at the pH given the difference spectrum against the blank of enzyme measured. The difference at 490 nm is plotted against pH (dotted line). Temp. 25°.

more the final difference spectrum is superimposed on the spectrum of free Tween 80.

The spectral changes induced in the enzyme by SDS are pH dependent. Fig. 22 shows that the binding of SDS to the enzyme is dependent on the ionisation of a group with a pK value around 6.6. The results indicate that a good correlation exists between the spectral shift induced and the decline in activity. In favor of the idea, that an ionised group is involved in the binding of SDS to the enzyme, is the dependence of the SDS inactivation on the ionic strength (Fig. 23). At higher ionic strength the inactivation of the enzyme by SDS is less pronounced; however the effect cannot be completely abolished.

It has been established (MASSEY and VEEGER, 1960) that NAD^+ is needed for the activity of this enzyme. After the enzyme has been treated with SDS, untill no spectral changes are induced anymore, NAD^+ cannot be bound to the enzyme, at least the spectral shifts characteristic for the binding of NAD^+ to lipoamide dehydrogenase (chapter 6) are not observed. Furthermore NAD^+ is not able to protect the enzyme against the influence of SDS (Fig. 21A). In the presence of NAD^+ the spectral shift at 390 nm induced by a certain SDS concentration is larger, while saturation is also reached at a lower SDS concentration. Furthermore the changes induced at 440 nm in the presence of NAD^+ start to occur at a lower SDS concentration as found in the experiment without NAD^+.

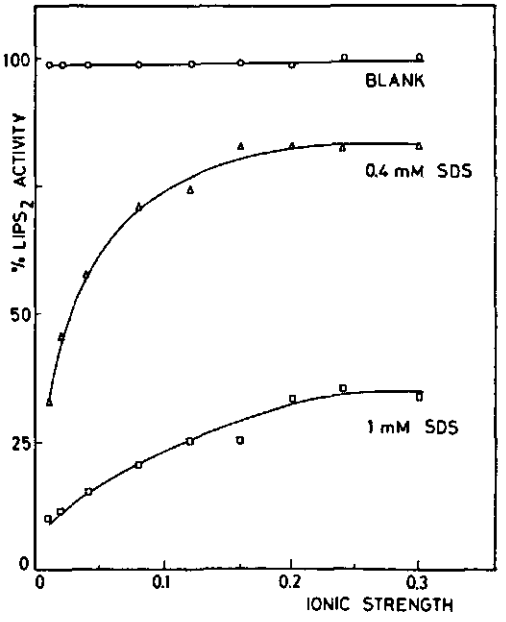


FIG. 23. The influence of the ionic strength on the effect of SDS on the activity of lipoamide dehydrogenase. The activity of enzyme (0.2 mg/ml), measured as in Fig. 1, in phosphate buffer pH 7.0 is plotted against the ionic strength at SDS concentrations: ○—○, no SDS; △—△, 0.4 mM SDS; □—□, 1 mM SDS. Temp. 25°.

The difference between the spectral shifts induced by SDS in the free enzyme and those in the enzyme-NAD^+ complex is at 390 nm larger and at 440 nm less than the spectral changes observed by binding of NAD^+ to the native enzyme at the corresponding wavelengths.

5.2.3. *Influence on the fluorescence properties*

The fluorescence of FAD, bound to the enzyme, decreases upon SDS binding to the enzyme, while the protein fluorescence increases (Fig. 24). Upon excitation at 300 nm the aromatic residues – mainly tryptophan (WEBER and YOUNG, 1964) – and the FAD chromophore are excited. The protein emission increases upon binding of SDS to the enzyme. At the same time the FAD fluorescence emission induced by excitation at either 300 nm or 365 nm (both isobestic points in the difference spectrum) both decline, but the quenching of the 300 nm excited FAD fluorescence is more pronounced on a relative base.

In Fig. 25 the polarization of the enzyme-bound flavin is plotted against the SDS concentration. At high SDS concentration the polarization approaches that of free flavin although it reaches this value only after 24 hours of incubation with SDS. Upon dialysis the flavin is removed immediately, while addition of free flavin has no effect.

Binding of Tween 80 does neither have influence on the fluorescence properties of the protein-bound flavin nor on the protein fluorescence. However since 300 nm was used as excitation wavelength, effects on chromophores at lower wavelengths, which are not expressed in these results, cannot be excluded.

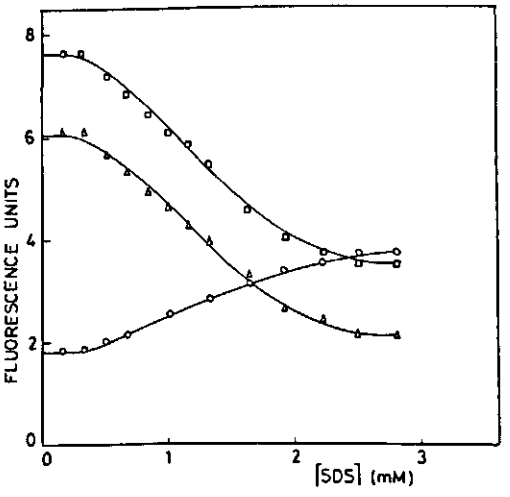


FIG. 24. Effect of SDS on the protein and flavin fluorescence of lipoamide dehydrogenase. Enzyme (0.15 mg/ml) in 30 mM phosphate buffer pH 7.2 Excitation and emission are plotted against the SDS concentration. Temp. 25°.
○—○, protein emission at 355 nm, excitation at 300 nm;
△—△, flavin emission at 520 nm, excitation at 300 nm;
□—□, flavin emission a 520 nm, excitation at 365 nm.

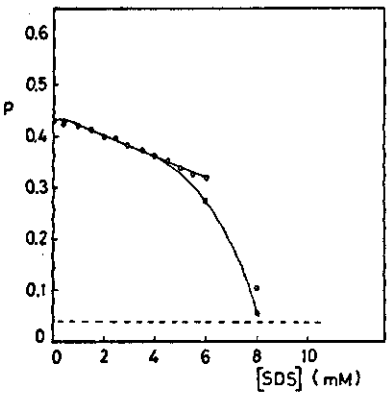


FIG. 25. Influence of SDS on the fluorescence polarisation of the 450 nm band of lipoamide dehydrogenase. Enzyme, (1.2 mg/ml) in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA, was incubated with several SDS concentrations and the polarisation measured at room temperature ○—○; immeadiatly after addition of SDS, ●—●; 24 hours after SDS addition.
Dotted line; fluorescence polarisation of free flavin.

The changes found in the absorbance and fluorescence spectra of the FAD chromophore and the protein are also reflected in the CD spectrum of the enzyme upon titration with SDS (Fig. 26). The spectrum of the SDS-saturated enzyme shows in the 250–400 nm region some of the characteristics of the CD spectrum of free flavin (MILES and URRY, 1968). Furthermore changes in the region of the amino acid bands at 250–300 nm can be observed. The dichroic bands due to the conformation of the enzyme in the 200–250 nm region did not change upon binding of SDS.

5.2.4. *Influence on hydrodynamic properties*

The fact that no changes are found in the apparent α-helix content of the enzyme upon SDS binding does not exclude the occurrence of association or dissociation phenomena (MCKENZIE et al., 1967). In Fig. 27A the behaviour of the sedimentation coefficient of lipoamide dehydrogenase upon binding of SDS

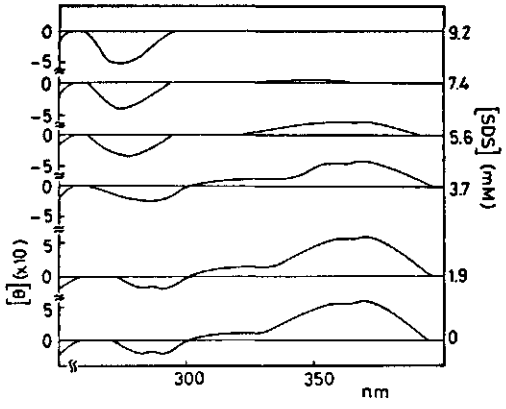


FIG. 26. Effect of SDS on the CD spectrum of lipoamide dehydrogenase. Enzyme (1.1 mg/ml) in 30 mM phosphate buffer pH 7.2 was treated with the concentration of SDS indicated and the spectra recorded. Temp. 25°.

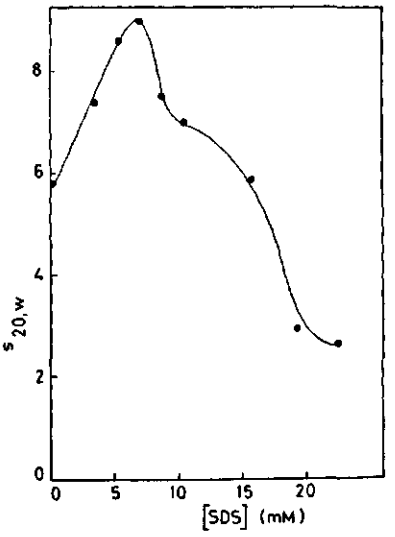


FIG. 27A. Influence of SDS on the sedimentation coefficient of lipoamide dehydrogenase. Enzyme (4 mg/ml) in 30 mM phosphate buffer pH 7.2 was treated with the concentrations SDS indicated and the average sedimentation coefficient of the sedimenting components was determined.

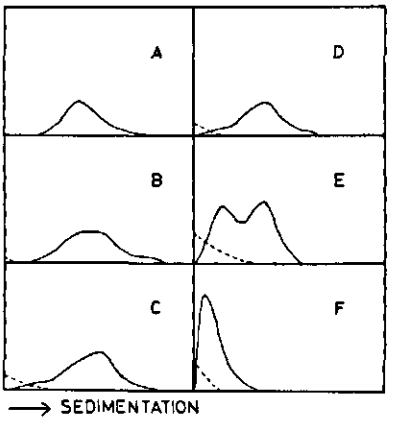


FIG. 27B. Influence of SDS on the sedimentation pattern of lipoamide dehydrogenase. Enzyme, 4 mg/ml, in phosphate buffer 30 mM, pH 7.2 containing 0.3 mM EDTA. Experimental conditions as given in methods. Pictures were taken at 22 minutes. Temperature 17°. A, control; B, 5 mM SDS; C, 7 mM SDS; D, 10 mM SDS; E, 16 mM SDS; F, 19 mM SDS.

to the enzyme is shown. In the reference cell buffer with the same SDS concentration was runned. In these cells clearly sedimentation of SDS could be observed, but in the sample cells no SDS peak could be detected. This suggests that SDS molecules are really bound or attached to the enzyme molecules (Fig. 27B). After an initial increase the sedimentation coefficient decreases to a constant value. The molecular weight of the species at high SDS concentration is 31,670. This value is lower than any other value found for the molecular weight of a modification of the enzyme. In the Svedberg relation used to calculate the sedimentation coefficient ideal behaviour is assumed and the value for the specific volume $\bar{v}$ is a value chosen from the literature. An uncertainity of 0.02 in $\bar{v}$ gives rise to a deviation of 10% in the sedimentation coefficient.

To see if a change in specific volume $\bar{v}$ occurred upon SDS binding to the enzyme, viscosity measurements were made. In Fig. 28 the kinematic viscosity is plotted against SDS concentration. The relation is not lineair and shows clearly that there are different processes. As all the SDS molecules are bound, the changes in the kinematic viscosity must be due to changes in size and shape of the protein molecule.

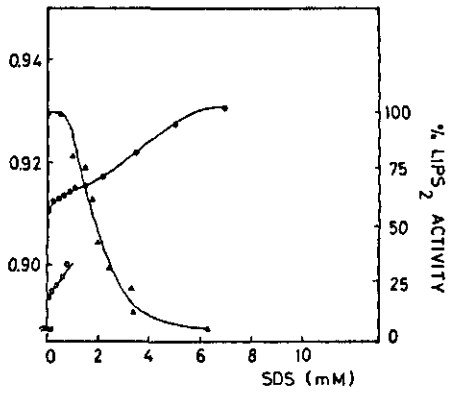


FIG. 28. Influence of SDS on the kinematic viscosity of lipoamide dehydrogenase.
○—○; buffer, 30 mM phosphate pH 7.2 containing 3 mM EDTA, with different SDS concentrations. At the arrow visible micelle formation occurred.
●—● ; enzyme, 1.1 mg/ml in 30 mM phosphate buffer pH 7.2 containing 3 mM EDTA, with different SDS concentrations. Each point is the average of ten measurements.
△—△; Loss of activity of enzyme (1.1 mg/ml) in 30 mM phosphate buffer, 30 mM pH 7.2 3 mM EDTA, with different SDS concentrations.
Temp. 25°.

The molecular weight determination by the use of gel chromatography is based on differences in size of molecules; density and shape are assumed to be constant. When differences in shape or density occur molecular weights calculated from these experiments are not reliable, unless for each change new calibration curves are made. Fig. 29 shows that the changes in elution pattern are de-

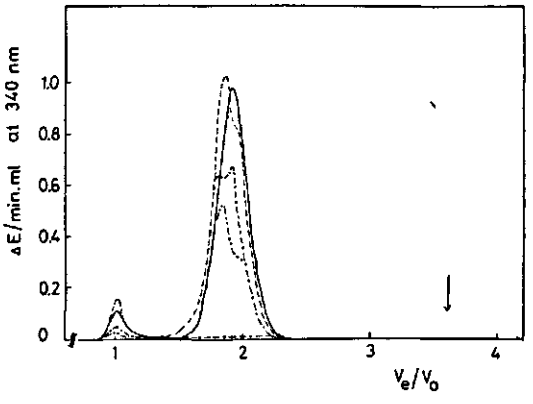


FIG. 29. Elutionpattern of the lipoate activity from a Sephadex G 200 column of lipoamide dehydrogenase in the presence of several concentrations of SDS. Enzyme, 2 ml of 4.4 mg/ml in 30 mM phosphate buffer pH 7.2 which was also the elutionbuffer, was brought on a column after incubation with;
——; no addition, – – –; 1 mM SDS, –.–.–; 4 mM SDS, ..–..–; 6 mM SDS, –.– –.; 20 mM SDS. At the position of the arrow an inactive protein peak is observed. The experiments were performed at room temperature.

pendent on the SDS concentration. It is difficult to detect the species with the high molecular weight due to the fact that part of the enzyme is bound to dextran blue and present in the void volume. Comparison of the patterns reveals that upon addition of SDS a larger amount of enzyme is present in the void volume. At higher SDS concentrations the species with the low molecular weight show up in agreement with sedimentation analysis. Activity measurements show that the component with the same elution volume as the native enzyme lost its activity in the presence of SDS, while the low molecular weight species never has any activity but still has FAD bound. The activity of the first component can be regained by putting the fraction on ice.

5.3. Discussion

Reynolds and Tanford (1970a) suggested that most proteins bind identical amounts of SDS on a gram basis and that most of the binding is of hydrophobic nature. The influence of the ionic strength on the binding of SDS to proteins is considered by them as due to an effect on the critical micelle concentration. As Tween 80, a non-ionic detergent, can be replaced by SDS on lipoamide dehydrogenase also in this case hydrophobic binding occurs and since after reaching a critical SDS concentration the binding and inactivation become dependent on the pH and the ionic strength we suggest that the initial binding phase occurs by means of hydrophobic interactions.

Although the dependence of the SDS binding on the ionic strength might be explained in terms of a shift in the critical micelle concentration, the dependence on the pH is not in agreement with this idea. Binding to or generation of an effect dependent on a charged group is a more likely explanation for the pH-dependency. With a pK value around 6.6 it is attractive to think of a hystidyl group since the dependence on the ionic strength shows that in this case the group involved must be of the kind $BH^+ \rightleftharpoons B + H^+$. However also lysine seems to be involved in the interactions of SDS with proteins in special cases (Braun and Radin, 1969). Tanford (1970) has pointed out that the side-chain of histidine is of partial hydrophobic character, this makes this side-chain less suitable for hydrophobic interaction with SDS. In agreement with the idea of ionic interactions between a charged group of the protein and the detergent is the dependence on the ionic strength. However the reactivation of the enzyme at low temperatures and the concomitant loss of the interactions between SDS and the enzyme point to a hydrophobic interaction. Another possibility is a change of the pK value of the charged group on the protein to a lower pH at low temperatures. Our observations that under our conditions no micelle formation can be observed at 20° disagrees with the idea that the effect of the ionic strength is an effect on the critical micelle concentration.

The ratio g SDS/g protein is for the initial binding phase 0.3. This value is somewhat lower than the value 0.4 found by Reynolds and Tanford (1970a) at low SDS concentrations.

A clear-cut interpretation of the second binding phase is not possible, two explanations can be offered 1: this phase is induced by ionic interactions 2: due to a shielding effect of the high concentrations of the poly-valent ion on the ionic interactions stronger hydrophobic interactions are induced which make higher concentrations of SDS necessary. Our results indicate that two kind of interactions occur c.f. the hydrophobic, not leading to a spectral shift and decrease in activity; the other leading to spectral shifts and decrease in activity. Furthermore the hydrophobic binding site must be occupied by SDS before the detergent can be bound to the binding site that is responsible for the pH-dependent effects. At saturation, that means total inactivation or maximal spectral shift, the value of g SDS/g protein is about the same as the value of 1.4 found by REYNOLDS and TANFORD (1970a) at high concentrations of SDS. It is likely that the enzyme binds only the monomeric form of SDS since binding still occurs at concentrations far above the critical micelle concentration.

The maximum amount of SDS bound, is lineairly related to the concentration of enzyme and the value mole SDS/mole enzyme is about the same in all methods used, except in the fluorescence studies where much lower SDS concentrations give a maximum effect. This may of course be due to the much lower enzyme concentrations used in the fluorescence experiments but another possibility is that this method shows only one of the different phenomena that occur. However no changes in fluorescence properties are observed upon binding of Tween 80 to the enzyme, so the hydrophobic binding site seems to have no influence on the fluorescence properties of the enzyme. The change in fluorescence indicates that part of the quenching of the enzyme-bound FAD emission upon excitation at 300 nm is due to abolishing of the energy transfer between tryptophan and flavin (VEEGER et al., 1971). The concomitant increase in protein emission is in agreement with this conclusion. The other part of the quenching of the flavin emission might be due to either a complex quenching by SDS or a local pertubation in the protein leading to interaction with the alloxazine ring or to the occurrence of isoalloxazine-adenine interactions.

The spectral titration curves support the idea that several processes of local conformational changes are induced by SDS binding. The maxima in the difference spectrum occur at different SDS concentrations. This suggests the presence of several ionic binding sites which exert a different pertubation effect on the vibronic bands of the FAD chromophore.

NAD^+ forms two spectrally visible complexes with the oxidised enzyme (chapter 6), an observation recently confirmed by SU and WILSON (1971) by means of fluorescence quenching studies. It has been postulated that protonation of one or more groups of the protein near N(1) of the isoalloxazine ring influences the spectral and redox properties of the flavin considerably (MÜLLER et al., 1970). The spectral properties of the NAD^+ complexes might be influenced in a similar way because the spectral shifts are probably induced by changes in polarity around the flavin (VISSER et al., 1969). As the spectral difference between SDS saturation with and without NAD^+ does not correspond with that of the complex between NAD^+ and the enzyme it seems that SDS does not re-

move NAD^+ from the enzyme once it is bound. This is in contrast to the fact that SDS binding prevents NAD^+ binding. These observations suggest a change of environment around the NAD^+ binding places. Although NAD^+ stays bound to the enzyme when the NAD^+-enzyme complex is treated with SDS the activity still declines. Thus the decrease in activity is not only due to a prevention of NAD^+ binding.

The CD data indicate that a change in local environment around the flavin is more likely to occur then a change in conformation of the protein. This means that SDS affects the hydrophobicity around the flavin. The fact that no changes in apparent α-helix content are observed support this conclusion. The changes observed in the CD spectrum around 260 nm are not necessarily due to changes of the protein conformation since the changes of the FAD conformation will also exert influence at this wavelength (MILES and URRY, 1968). Furthermore the changes in the CD spectra and the fluorescence polarisation suggest that the binding of FAD to the enzyme at SDS saturation is less tightly, a situation also suggested by MASSEY for the fully reduced enzyme (1963) and by CASOLA et al. (1966) for the Cu^{2+}-treated enzyme. This is confirmed by the fact that dialysis can remove the FAD from the enzyme at SDS saturation. This weakening and in the end loss of FAD binding is accompanied by a decrease in energy transfer from the protein to the prosthetic group in the case of SDS titration. The increase in fluorescence yield of the aromatic amino acid residues does not need to be entirely due to this decrease in energy transfer to the flavin, but can also be due to a decrease in the quenching of this fluorescence by the protein itself due to slight changes in the protein conformation. It has been found that in the holoenzyme only 30% of the expected tyrosine and 10% of the expected tryptophan fluorescence can be observed; furthermore the apoenzyme has an increased fluorescence (VISSER and VEEGER, 1970). The influence of SDS on the enzyme is not completely reversible, which might be due to fact that a part of the weaker bound FAD is liberated from the enzyme during the SDS treatment.

Monomerisation occurs as can be seen from the sedimentation and chromatografic behaviour of the enzyme. Monomerisation is not in contradiction with ORD and CD results as the dimer, the monomer and the apoenzyme have apparent α-helix content of respectively 30%, 20% and 60%. All the effects can level each other.

The value of 31,500 found for the molecular weight of the monomer is considerably lower than the expected value of 52,000, the second value however has been corrected for changes in shape. COHN et al. (1971) find even a lower value of 25,000. They used the method recommended by REYNOLDS and TANFORD (1970b), SDS gel-electrophoresis and chromatography on Sephadex G-200 with the protein in urea and 2-mercaptoethanol.

Changes in specific volume can be due to changes in hydratation, a possibility also suggested by the fact that difference spectrum of bovine serum albumin with SDS is the same as that of BSA with sucrose or ethylene glycol (RAY et al., 1966). It can also be due to the formation of a shell of SDS molecules around the enzyme molecule.

Another possibility to explain the anomalous behaviour of the enzyme-SDS complex is a large charge effect, since it has been shown that all protein-SDS complexes, even of proteins with a high iso-electric point (pH 11) move to the anode in electrophoresis experiments (COWGILL, 1964). These charge interactions can be the cause of the presence of the '$s_{20,w} = 8.9$' species.

FISH et al., (1970) concluded from viscosity measurements that all protein-SDS complexes – denatured and reduced – have the same shape, a rod or ellipsoid. Lipoamide dehydrogenase belongs to the class of globular proteins. The behaviour of the kinematic viscosity also suggests, by the slope of the plot, that the enzyme-SDS complex has a more asymmetrical shape. However at higher concentrations of SDS the decrease of the slope suggests the return to a more symmetrical shape of the complex possibly due to monomerisation.

The dissociation of the protein-SDS complex is dependent on the pH, a situation similar to the dissociation of the enzyme upon dilution (VISSER, 1970). Also the monomer obtained by dilution splitts off its flavin after several days (chapter 4).

6. BINDING OF NAD^+ TO LIPOAMIDE DEHYDROGENASE

6.1. INTRODUCTION

MASSEY and VEEGER (1961) reported the requirement of NAD^+ for the oxidation of NADH with lipoic acid by lipoamide dehydrogenase from pig heart. This requirement was confirmed for the enzyme isolated from other species (MASSEY, 1963). It was postulated that NAD^+ excerts its function by preventing the conversion of the enzyme by excess NADH into the inactive fully reduced – by four equivalents – form. The active center is capable of reacting with two moles of NADH, provided that NAD^+ is removed, resulting in the fully reduced state. In the presence of NAD^+ the reaction with the second mole of NADH is effectively prevented, except at low temperatures where further reduction is favoured. Excess reduced lipoic acid does not lead to the fully reduced state of the enzyme in the absence of NAD^+ but a stable two equivalent reduced state, the so called 'semiquinone', is formed.

MASSEY et al. (1960) proposed a mechanism in which the NAD^+, bound to a SH-group of the active center, prevents the enzyme from four-equivalent reduction. However after modification of the two equivalent reduced enzyme by reaction of arsenite with the reduced disulfide bridge in the active center, the addition of NADH results in the formation of the four equivalent reduced form via the formation of intermediates with similar spectral characteristics as those of the two equivalent reduced form (VEEGER et al., 1966). Spectral evidence for NAD^+ binding comes from the observation that the spectra of the two equivalent-reduced forms after reduction either by $lip(SH)_2$ or NADH are not completely identical. The spectrum after reduction with NADH shows an enhanced absorption band at 530 nm over the spectrum obtained by $lip(SH)_2$ reduction. This extra absorption at 530 nm disappeared upon NAD-ase treatment and was identified as belonging to the NAD^+-bound complex. VEEGER and MASSEY (1961) observed that NAD^+ bound to the two equivalent reduced enzyme converts the so called 'semiquinone' spectrum into a flat absorption band in the 500–600 nm region. VEEGER et al. (1970) found that this species rather than the 'semiquinone' is the active intermediate in the reduction of NAD^+ by reduced lipoate. Upon four equivalent reduction of the enzyme with NADH in the presence of arsenite a broad absorption band with a maximum at 750 nm appears, giving the enzyme a blue-green color. This absorption band can also be abolished by NAD-ase and was identified to belong to a charge transfer complex between NAD^+ and $FADH_2$. A sulfhydryl group was proposed to be involved in the binding of NAD^+ (VEEGER and MASSEY, 1963).

In the activity measurements the influence of NAD^+ is shown by the abolishment of the lag period in the oxidation of NADH by lipoic acid by the enzyme. When NAD-ase is incubated in the reaction mixture the reaction is almost completely inhibited. Replacement of the amide side group of the pyridinium ring of

NAD^+ by an acetyl or aldehyde group results in the loss of activity (MASSEY, 1963).

This activating function of NAD^+ is also found for lipoamide dehydrogenase isolated from other sources. In case of the enzyme isolated from *Spinacea oleracea* addition of NAD^+ results in a shift of the pH optimum of the oxidation of NADH with lipoamide from pH 7.0 to pH 6.3 (MATTHEWS and REED, 1963). SEARLS and SANADI (1961) observed in their studies with lipoamide dehydrogenase isolated from the α-ketoglutaric dehydrogenase complex that the lag period in the oxidation of NADH with lipoate could be eliminated by NAD^+, but at pH values above 6.3 no lag period was observed.

VISSER (1970) concluded from kinetic studies that the best explanation of the phenomena observed was given by a preferred order ternairy complex mechanism although he did not exclude the possibility of negative cooperativity. STEIN and CZERLINSKI (1967) have reported that at high NAD^+ concentrations nucleotide complexes with lipoamide dehydrogenase are formed, while furthermore the pyridine nucleotide is a competitive inhibitor with respect to NADH in the reduction of lipoic acid derivatives. SU and WILSON found in agreement with our results, that two pairs of NAD^+ binding sites exist (1971). VAN DEN BROEK and VEEGER (1972) found complexes of pyridine nucleotides with transhydrogenase from *Azotobacter vinelandii* while also in this case the nucleotide was an inhibitor. Glutathione reductase is also able to form complexes with $NADP^+$ which are competitive inhibitors of the reduction of reduced glutathione by NADPH (STAAL et al. unpublished results).

It was thus of interest to investigate whether these spectral complexes could be correlated with the obligatory presence of NAD^+ in the lipoate activity.

6.2. RESULTS

6.2.1. *Difference spectra*

The difference spectra obtained upon addition of NAD^+ to oxidised lipoamide dehydrogenase are shown in Fig. 30. It is clear that the difference spectra obtained at 25° and 5° after addition of 1–2 mM NAD^+ to the enzyme differ not only in magnitude but also in shape. At these saturating NAD^+ concentrations at both temperatures two positive maxima at 507 nm and 387 nm and four negative maxima at 477 nm, 450 nm, 430 nm and 370 nm are visible. STEIN and CZERLINSKI (1967) did not mention the occurrence of the 477 nm maximum, but detected the other maxima. The 450 nm maximum becomes visible only at NAD^+ concentrations higher than 0.1 mM, while at 5° even at saturating NAD^+ concentrations its contribution to the difference spectrum is less than at 25°. Of interest is the occurrence of the broad maximum around 320 nm at 0.1 mM NAD^+, which is replaced by a continuous absorbance increase into the ultraviolet at higher NAD^+ concentrations.

In order to calculate the number of molecules of ligand bound to the enzyme the method of STOCKELL (1959) was applied. Fig. 31 shows the results of the titration of the enzyme with NAD^+, while Fig. 32 shows the corresponding

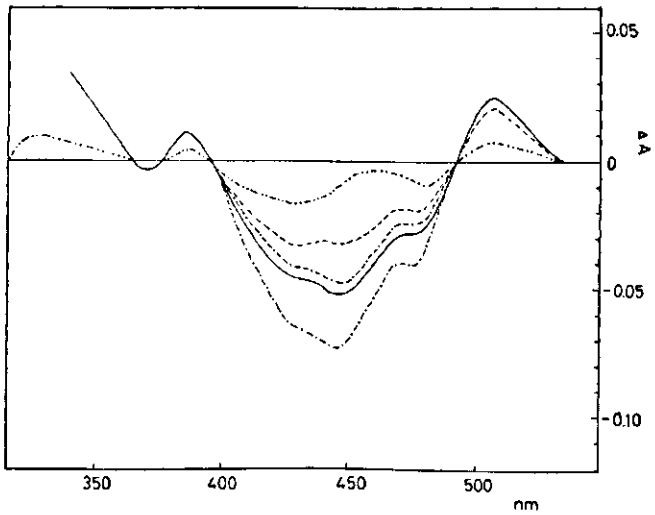


FIG. 30. Effect of NAD^+ on the spectrum of lipoamide dehydrogenase. Difference spectra of enzyme (8 mg/ml) plus NAD^+ minus enzyme in 30 mM phosphate buffer pH 7.2. ——, 1.4 mM NAD^+ at 25° (saturating); – – –, 1.0 mM NAD^+ at 5° (saturating); –..–.., 0.1 mM NAD^+ at 5°. Difference spectra measured in tandem cells, cell 1, enzyme plus NAD^+ plus lipoamide and cell 2 buffer minus cell 1 enzyme and cell 2 buffer plus NAD^+ plus lipoamide: –.–.–, 1.4 mM NAD^+ plus 1.4 mM lipoamide at 25°; – –.– –., 1.0 mM NAD^+ plus 1.0 mM lipoamide at 5°.

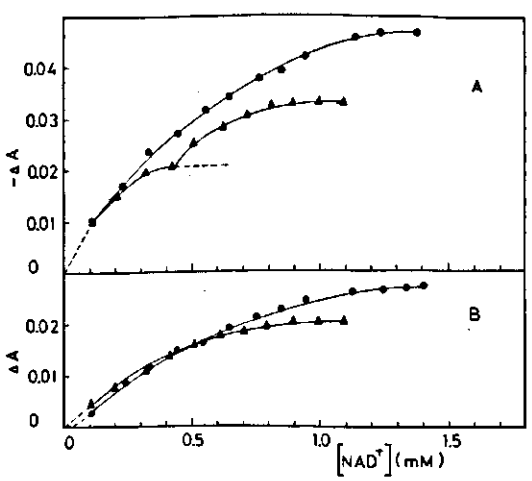


FIG. 31. Spectral titration curves of lipoamide dehydrogenase with NAD^+. For conditions see Fig. 1. Absorbancy differences: A, at 430 nm; ●—● at 25°; ▲—▲ at 5°; B, at 507 nm; ●—● at 25°; ▲—▲ at 5°.

Stockell plots. The saturation curve at 430 nm is biphasic at 5°, while at 25° such an irregularity is not observed. The plots in Fig. 32 indicate that simple saturation curves are not obtained. The peculiar Stockell plots from the data at 507 nm at 25° can be explained by the S-shape of the titration curve at this wavelength. Also the Stockell plot from the titration curve at 430 nm at 25° shows that at this wavelength not a true hyperbolic titration curve is obtained. From these results and the change in ratio A_{430}/A_{450} during the titration can be suggested that the enzyme contains different NAD^+ binding sites. It is reasonable to assume that these are pairs of two equal binding sites because the enzyme contains two independent FAD molecules (MASSEY and VEEGER, 1961 and MASSEY et al., 1960). Thus at 5° the first maximum at 430 nm could represent the saturation of the first pair of NAD^+ binding sites. This assumption is supported by the lineairity of the Stockell plot in this concentration range, which gives two binding sites per mole of protein with $K_{diss} = 35$ μM (Fig. 32A). The analysis at other wavelengths shows that at 5° at 477 nm and 507 nm lineair Stockell plots are obtained over the whole concentration range, from which four binding sites per mole of protein with an overall $K_{diss} = 90$–110 μM can be calculated (Fig. 32C). At 450 nm and 387 nm nonlineair Stockell plots are obtained at 25° and 5°, from these non-lineair plots four binding sites can be estimated at higher NAD^+ concentration.

Titration of the enzyme with lipoamide did not result in any spectral difference in the wavelength region measured. In the presence of NAD^+ concentrations

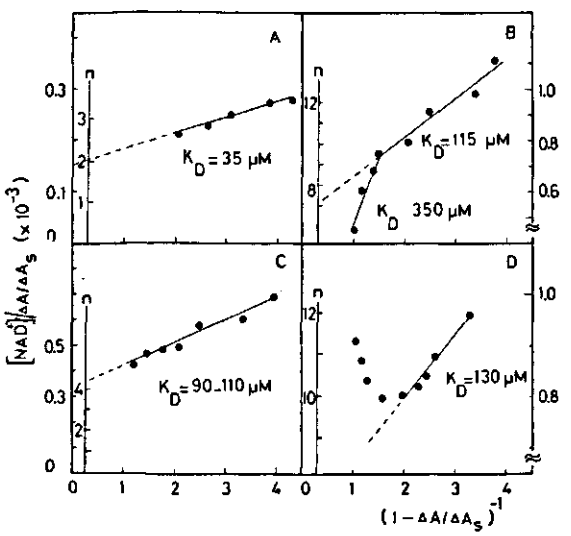


FIG. 32. Determination of the number of NAD^+ binding sites by the method of Stockell (1954) Data from Fig. 2. A, 430 nm at 5°, number of sites calculated from the first plateau in the titration curve; B, 430 nm at 25°, total curve; C, 507 nm at 5°, total curve; D, 507 nm at 25°, total curve.

less than 0.1 mM, which does not give the 450 nm band in the difference spectrum, lipoamide has also no effect in this wavelength region. At saturating NAD^+ concentration the addition of lipoamide results in a further increase of the spectral differences without any shift in wavelength (Fig. 30). Also in this case the effect is dependent on the temperature, i.e. after the addition of lipoamide at 25° the ratio A_{450}/A_{430} remains the same but at 5° the 450 nm maximum increases more and the difference spectrum resembles in shape and magnitude the difference spectrum without lipoamide at 25°.

The flavin fluorescence is very little quenched upon binding of NAD^+ to the enzyme, less than 6%, however SU and WILSON (1971) used this quenching to determine the number of binding sites for NAD^+.

For the Cu^{2+}-treated enzyme the difference obtained upon the addition of NAD^+, declines proportionaly with the decreasing NADH-lipoate reductase activity.

The difference spectrum obtained upon addition of NAD^+ to the enzyme is pH dependent (Fig. 33). However at all pH's the biphasicity of the titration curves is observed. The difference spectrum at pH 5.2 at 25° is very similar with that at pH 7.2 at 5°. The ratio A_{450}/A_{430} is about the same at both pH's. At pH 7.8 this ratio changes, the maximum at 450 nm being less pronounced and the cross-over point shifted to shorter wavelength.

Also the ionic strength has influence on the titration curves of the enzyme with NAD^+. At increasing ionic strength the affinity of NAD^+ for the first binding place increases. This effect of the ionic strength is also observed in the pH effect upon NAD^+ binding to the enzyme. At pH 5.6 $K_D = 50\ \mu M$ at I = 0.1 while at I = 0.2 $K_D = 35\ \mu M$.

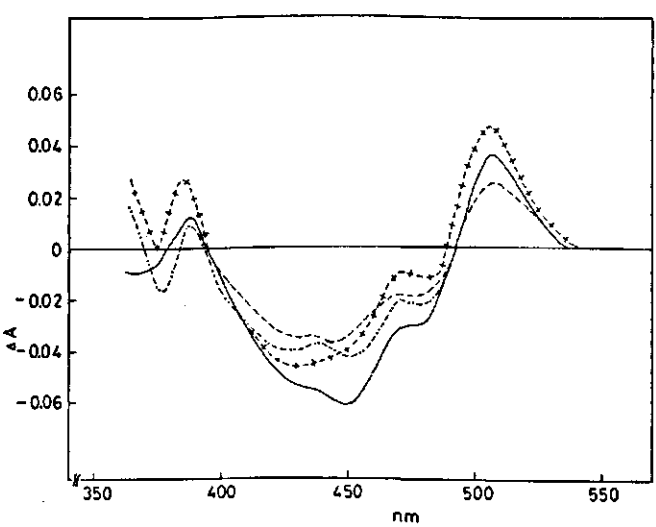


FIG. 33. The pH dependency of the NAD^+ binding difference spectrum. For conditions see Fig. 1. Ionic stenght 0.07 Difference spectra at saturating NAD^+ concentrations at ——, pH 7.2; + + +, pH 7.8; –.–.., pH 5.2 at 25° and – – –, pH 7.2 at 5°.

6.2.2. *Molecular weight of the enzyme-NAD$^+$ complex*

Sephadex G-200 experiments were performed to investigate whether NAD$^+$ binding has influence on the monomer-dimer equilibrium. However NAD$^+$, under the conditions used, phosphate buffer pH 5.2–7.8 and ionic strength 0.01–0.07, showed affinity for the Sephadex G-200. The enzyme was eluted from the column without any bound NAD$^+$, while NAD$^+$ was eluted in a fraction of which the volume was much larger than the total volume of the column. Determination of the molecular weight by ultracentrifugation showed one species in the dimeric form at NAD$^+$ concentrations of 0.1 mM and 1 mM. Light-scattering revealed that NAD$^+$ even shifts the monomer-dimer equilibrium to the dimeric side. From a preparation able to dissociate upon dilution the molecular weight decreases with decreasing protein concentration as previously shown by VISSER (1970). The same preparation incubated with NAD$^+$ shows little dependency of $\overline{M}_{app}$ on the enzyme concentration, even at low protein concentration $\overline{M}_{app}$ increases. The experiment was done at 0.1 mM and at 1 mM NAD$^+$, both giving the same result (Fig. 34). These results suggest that the first two moles of NAD$^+$ bound per mole of enzyme (100,000: molecular weight), keep the enzyme in the dimeric form. This result is in accordance with the influence of NAD$^+$ on the lipoate activity of diluted enzyme preparations (chapter 3).

6.2.3. *Effect of NAD$^+$ on the lipoate activity of the enzyme*

According to MATTHEWS and REED (1963) the pH optimum of the NADH-lipoate reduction by the enzyme isolated from *Spinacea oleracea* is shifted in the presence of NAD$^+$ from pH 7.0 to pH 6.3. The pH-activity curves of the NADH-

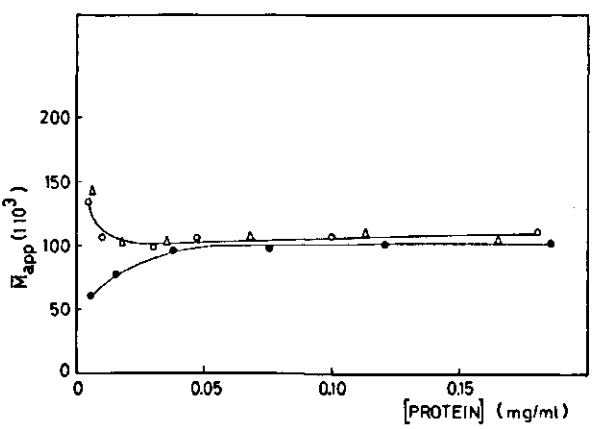


FIG. 34. Dependency of $\overline{M}_{app}$ on the protein concentration in the absence and presence of NAD$^+$. ●—●, no NAD$^+$; ○—○, 0.1 mM NAD$^+$; △—△ 1.0 mM NAD$^+$.

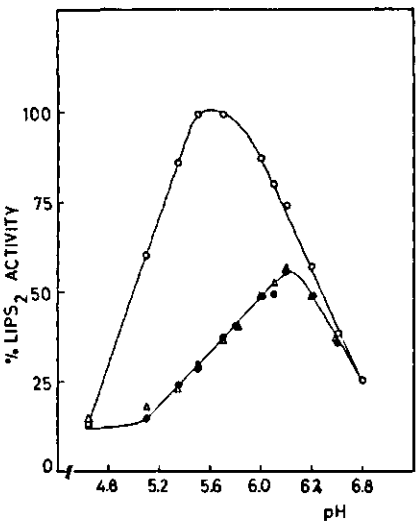


FIG. 35. The pH- activity curves of the NADH-lipoate reduction by lipoamide dehydrogenase. Activity was measured as given in the methods. In the reaction mixture the addition of NAD^+ was ommitted and the following additions were made. O—O, in the presence of 0.1 mM NAD^+; Δ—Δ, in the presence of 0.2 mM phosphate, ●—●. without NAD^+.

lipoate reduction by the pig heart enzyme were determined under different conditions; Fig. 35 shows the results. In this case also a shift to lower pH of the optimum in the presence of NAD^+ is observed. In the presence of NAD^+ a bell-shaped activity curve is observed. The pH optimum was also determined in the absence of NAD^+ but with phosphate ions added to the reaction mixture.

These sets of curves can be simulated. When we assume that the active center is largely determined by the ionization of two groups, we can according to DIXON and WEBB (1964) compute pH-activity curves by assuming the pK values for the two group in the active center. For this enzyme one pK value was taken to be 6.6, based on SDS titrations (chapter 5) and the other pK value was assumed to be shifted by NAD^+ from pH 6.2 to 4.9. Sets of curves are obtained for the four possibilities. The experimental curves are closely approaching the situation in which it is assumed that $E\begin{smallmatrix}A^- \\ BH\end{smallmatrix}$ is the active species while $E\begin{smallmatrix}A^- \\ B^-\end{smallmatrix}$, $E\begin{smallmatrix}AH \\ B^-\end{smallmatrix}$ and $E\begin{smallmatrix}AH \\ BH\end{smallmatrix}$ are inactive. The shift in the pH optimum upon addition of NAD^+ can be achieved by shifting the pK value of the deprotonated group to a lower pH (Fig. 36). Although the experimental curves and the computed curves show a close resemblance, there are differences. These differences indicate that the active center is not only determined by these two groups but that also other interactions excert their influence on the active center. Another possibility is that, as indicated by the result that the species $E\begin{smallmatrix}AH \\ BH\end{smallmatrix}$ is not completely inactive. The best fit of the experimental curves with the computed curves is obtained by assuming the active species with a protonated group with a pK 6.6 and a deprotonated group with pK 6.2 whose pK value is shifted upon NAD^+ binding to 4.9–5.0. See also Fig. 35.

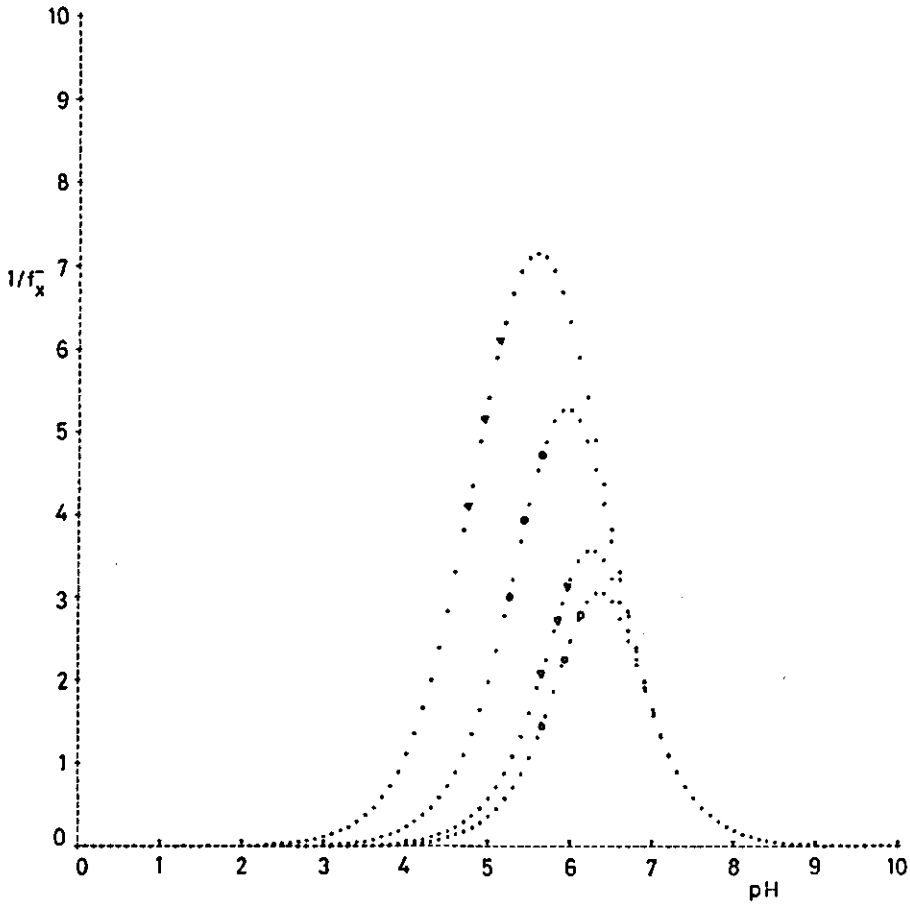


FIG. 36. Computed pH-activity curves for a system with active center $E\langle^{BH}_{AH}$ Plotted $1/f_x^-$ against pH (DIXON and WEBB, 1964)

$$f_x^- = 1 + \frac{K_{1y}}{K_{1x}} + \frac{[H^+]}{K_{1x}} + \frac{K_{2y}}{[H^+]}$$

$1/f_x^-$ gives the fraction of the total amount which is present in the state $E\langle^{BH}_{A^-}$. $K_{2y} = K_{1y}$ is kept constant at $10^{-6.6}$; values of K_{1x}: ○—○, $10^{-6.4}$; △—△, $10^{-6.2}$; ●—●, $10^{-5.6}$; ▲—▲, $10^{-4.9}$.

6.2.4. *pH effect of NAD$^+$ binding to the enzyme*

Enzyme was extensively dialysed against 0.2 M NaCl pH 5.7 to remove all phosphate ions (chapter 3). When the pH of the enzyme solution was measured with the pH-meter a gradual increase in pH was observed, presumably due to adsorption of the enzyme to the membrane of the glass electrode. For this reason

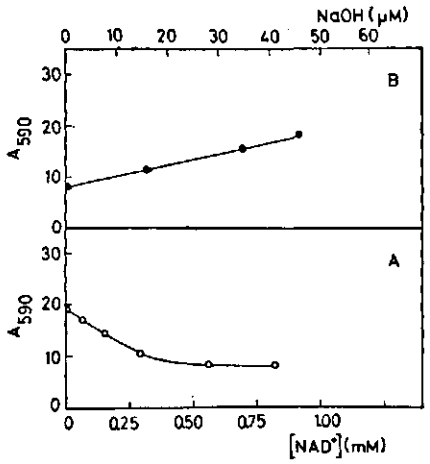


FIG. 37A. The pH titration curves of lipoamide dehydrogenase with NAD^+. Enzyme concentration, after extensive dialysis against 0.2 M NaCl, pH 5.7, 2.6 mg/ml Temperature 25°. To all solutions used in this experiment 10μg bromocresolpurple per 2 ml solution was added. NAD^+ dissolved in 0.2 M NaCl and titrated to pH 5.7 with NaOH, was added in the concentration indicated to a final volume of 2 ml enzyme solutions. Tandem cells were used. A_{590} is plotted against NAD^+ concentration.

B. Determination of the number of protons liberated upon NAD^+ binding. Spectral difference at 590 nm, from enzyme solutions as obtained in Fig. 8A upon the addition of 0.83 mM NAD^+, was titrated back to its original absorbance with 0.906 mM NaOH, to which 10μg bromocresolpurple per 2 ml was added. Increase in A_{590} is plotted against NaOH concentration.

the titration experiments were carried out in the presence of an indicator, bromocresolpurple, which changes color between pH 5.2 and pH 6.8 from yellow to purple. At the basic side the indicator shows an absorption spectrum with maxima at 590 nm and 365 nm, at the acid side only a maximum at 420 nm while no absorbance at 590 nm is present in the absorption spectrum. At 590 nm no interference with absorption of enzyme or enzyme-NAD^+ complex occurs. At this wavelength the pH changes due to the NAD^+ binding to the enzyme were studied. Fig. 37A shows the decrease in absorbancy at 590 nm upon addition of NAD^+ to the enzyme, indicating that the pH decreases and protons are liberated upon NAD^+ binding. Addition of NAD^+ will buffer the solution, therefore the difference in absorbancy at 590 nm was titrated back with diluted NaOH solution until the original absorbancy (Fig. 37B). The result shows that 0.9 mole NaOH per mole of FAD has to be added at NAD^+ saturation, suggesting that upon NAD^+ binding to the enzyme one proton per FAD is liberated.

Comparison of the pH titration curves with the spectral titration curves shows that the pH effect occurs during the binding of NAD^+ to the binding site with the highest affinity.

6.3. Discussion

The spectral shifts induced in the oxidised enzyme by NAD^+ confirms the suggestion by Massey and Veeger (1961) that two NAD^+ complexes with the enzyme exist; at NAD^+ concentrations less than 0.1 mM a complex is visible which has similar spectral characteristics as the complex at saturating NAD^+ concentrations except that the negative maximum at 450 nm is absent. The difference spectrum reflects a pertubation around the flavin binding site. However the enzyme shows also in the flavin absorption wavelength region small temperature-dependent changes (Mayhew et al., 1972) in the 2–30° region. Although these temperature effects may have some influence on the difference spectra upon NAD^+ binding to the enzyme, they cannot account for the total difference. The difference in magnitude of the 507 nm maximum at 25° and at 5° in the NAD^+-binding difference spectrum is small (0.001 difference in absorbance per mg of enzyme). The temperature-induced spectral shift induces in the difference spectrum of the 300–500 nm region a maximum at about 507 nm, which is the largest in magnitude (0.005 difference in absorbance per mg of enzyme). This suggest that the difference in absorbance between NAD^+ binding at 25° and 5° could only for a small part be due to the temperature pertubations or be due to a difference in pK value of the group involved.

From the spectral shifts induced by lipoamide in the presence of NAD^+ it is clear that the lipoamide forms a ternary complex with the enzyme which has NAD^+ bound to the site with the lowest affinity, which is thus likely to be the catalytic site. This is supported by the kinetic data from Visser (1970) and Stein and Czerlinski (1967), that the inhibition of the NADH oxidation by NAD^+ has a $K_i = 0.2$ mM. The complex with NAD^+ bound to the highest affinity site is thus the enzyme with the regulatory NAD^+ molecule bound. As very little quenching of the flavin fluorescence occurs, the difference spectrum is probably not due to complex formation but to changes in environment of the flavin. The spectral shifts induced at pH 5.2 at 25° is very similar with that at pH 7.2 at 5°. At low temperature the reduction state of the NADH reduced enzyme shifts towards the four equivalent reduced state even in the presence of NAD^+ (Massey and Veeger, 1961), this can also be expected at low pH and has been actually observed (Veeger, unpublished results). This suggests that at low pH and at low temperature the binding to the regulatory NAD^+ binding site is much less effective.

Activating effects of low concentrations product are observed with other enzymes, for instance glyceraldehyde-3-phosphate dehydrogenase (Hilvers et al., 1964), beef liver glutamic dehydrogenase (Olson and Anfinsen, 1953) and human heart lactic dehydrogenase (Nisselbaum and Bodanski, 1961). Conway and Koshland (1968) suggested that the activating effects of products on the enzyme activity can be ascribed to negative cooperativity. Visser (1970) concluded that lipoamide dehydrogenase acts according to a preferred order ternary complex mechanism, although there were no objections against negative cooperativity. However with this enzyme two distinct different binding

sites exist with different spectral properties and two different affinities can be calculated from the difference spectra. This suggests that binding of the first NAD^+ molecule induces a change in conformation in one subunit but has no effect on the conformation of the other subunit.

TRENDHAM (1968) studied the pH-dependency of the NAD^+ binding to glyceraldehyde-3-phosphate dehydrogenase. He found a pH dependent absorption band in the 300–400 nm region upon NAD^+ binding to the enzyme and observed a pK value of 5.4 for the group involved, a SH-group forming a charge-transfer complex with NAD^+. Trendham suggested that upon NAD^+ binding to the enzyme the pK value of an essential SH-group is shifted to lower pH. Also in the case of lipoamide dehydrogenase a change in pK value of an essential group can be correlated with the binding of NAD^+ to the enzyme. Also in this case a sulfhydryl group might be involved.

When the SH-groups are blocked by PCMB, NADH cannot reduce the FAD; furthermore the two equivalent-reduced enzyme is not stable when PCMB (MASSEY, 1963) or iodoacetamide (STEIN and STEIN, 1972) is added. Also the fact that the Cu^{2+}-treated enzyme which contains two SH-groups less, shows no spectral shift upon the addition of NAD^+ indicates the involvement of a sulfhydryl group. It does not exclude the possibility that NAD^+ is still bound to the Cu^{2+}-treated enzyme because the spectral shift is generated by a pertubation of the FAD environment, while FAD is bound in a more polar environment in the Cu^{2+}-treated enzyme (VEEGER and MASSEY, 1961). The fact that the Cu^{2+}-treated enzyme yields upon reduction with NADH the blue-green band with the maximum at 700 nm due to a charge-transfer complex between $FADH_2$ and NAD^+ indicates that NAD^+ still can be bound to the reduced enzyme. The nucleotide cannot excert its stabilising function, probably because it is bound to the catalytic NAD^+ binding site. The observation that no spectral shift is observed in the presence of NAD^+ after Cu^{2+}-treatment may indicate that this shift is generated by the pertubation of the interaction between the flavin and a SH-group. DE KOK et al. (1968) suggested that the flavin fluorescence is probably quenched by a SH-group in the vicinity of the flavin.

The fact that NAD^+ favors the dimeric state, together with the observation that its obligatory role in the NADH-lipoate reduction diminish at higher pH and even disappears above pH 6.5 (Fig. 6) explains some of the different results obtained by different laboratories. Apoenzyme prepared by acid-treatment according to KALSE and VEEGER (1968) is a monomeric species while apoenzyme prepared at pH 7.6 – where NAD^+ is not needed in the assay – yields a dimeric species (MASSEY, private communication). STEIN and STEIN (1972) recently stated that NAD^+ potentiates the reduction of lipoate by NADH by preventing dissociation of the enzyme into inactive components, a result in agreement with our results and indicating that the dimer is the active species, which is in accordance with the results obtained by VISSER (1970) with the enzyme dissociating upon dilution. It seems that the stabilising role of NAD^+ is to keep the enzyme in the dimeric form, as a consequence of the shift in pK value of a group. VISSER (1970) observed, that under conditions where the four equivalent-reduced en-

zyme is obtained, dissociation into monomers occurs. Together with our results one might suggest that the dimer cannot be reduced beyond the two equivalent-reduced state.

From these results can be suggested that the activity of lipoamide dehydrogenase is largely determined by the presence in the active center of a group with pK value 6.6 (chapter 5) in the protonated state, probably a histidine or a lysine, and by the presence of a group with pK value 6.2 that is shifted to 4.9–5.0 upon NAD^+ binding, presumably a sulfhydryl group, which has to be in the deprotonated state in the active form. This shift in pK value is accompanied by a shift in the monomer-dimer equilibrium to the dimer side. NAWA et al. (1960) found for the enzyme isolated from *Escherichia coli* that the lipoic acid was bound by its carboxylgroup to an ε-amino of a lysine residue in the protein, while WILLIAMS and ARSCOTT (1972) identified a lysine residue in the peptide sequence around the active center cystine of the enzyme isolated from *Escherichia coli* as well as from pig heart. These results suggest a lysine as an active group for the pig heart enzyme.

SUMMARY

The existence of a monomer-dimer equilibrium with lipoamide dehydrogenase is demonstrated. The equilibrium can be shifted to the monomer side at low ionic strength and low pH by removing the phosphate ions by extensive dialysis. At low ionic strength, I : 0.01 and 0.02, the enzyme precipitates while aggregation takes place. This aggregation seems to be due to changes in the activity coefficient of the enzyme. High phosphate concentrations, NAD^+ and high temperatures favor association. Also bringing the enzyme in a more polar environment causes dissociation. Dioxan and 2-chloroethanol are used to decrease the dielectric constant of the buffer solution. Inactivation and dissociation of the enzyme is time-dependent in these solutions. High concentrations of dioxan and 2-chloroethanol cause denaturation and precipitation of the enzyme. High phosphate concentrations stimulate the denaturation and precipitation of the enzyme in dioxan and 2-chloroethanol.

Dissociation of the enzyme is accompanied by loss in activity and decrease in apparent α-helix content. ORD and CD data show this decrease, however the possibility that this decrease is due to changes in shape and size of the protein molecule cannot be excluded. Fluorescence and CD experiments show that upon dissociation an amino acid, a tryptophan residue, moves to a more polar environment. Also by treating the enzyme with dioxan a tryptophan residue is pertubed.

Dissociation of the enzyme can also be achieved by treating the enzyme with sodium dodecylsulfate. Hydrophobic and ionic interactions are observed. Binding to the hydrophobic sites, by sodium dodecylsulfate or Tween 80, has no influence on the lipoate activity and on absorption spectrum of the enzyme in the visible-region. Binding to the ionic sites causes loss in lipoate activity and affects the absorption spectrum. From the dependency on the pH and the ionic strength it is concluded that a group of the kind $BH^+ \rightleftharpoons B + H^+$ with a pK value around 6.6 is involved. At high SDS concentrations the binding of FAD to the enzyme is weakened and upon standing for long times the flavin dissociated off.

Dimerization of the enzyme is favored by NAD^+. Binding of NAD^+ to the enzyme yields a difference spectrum. From these spectral titration curves two pairs of NAD^+-binding sites are calculated, the binding site with the highest affinity, $K_{diss} = 35\ \mu M$ is assigned to the regulatory site while the binding site with $K_{diss} = 90\text{–}110\ \mu M$ is assigned to the catalytic site. Upon NAD^+ binding to the regulatory site one proton per FAD is liberated. Comparision of the pH activity curves with computer models shows that the activating effect of NAD^+ in the lipoate activity can be explained by a shift in pK value of a group from pH 6.4–6.3 to 5.0–4.9 upon NAD^+ binding. Together with observations in the literature these results suggest that the pK value of a SH-group is shifted to lower pH upon NAD^+ binding. This SH-group is suggested to be functional in the S^- state in the active center.

SAMENVATTING

Het bestaan van een monomeer-dimeer evenwicht in lipoamide dehydrogenase is aangetoond. Het evenwicht kan worden verschoven in de richting van het monomeer door de phosphaat ionen te verwijderen door extensive dialyse bij lage pH en lage ionsterkte. Bij lage ionsterkte, I = 0,01 en 0,02, slaat het enzym neer terwijl aggregatie optreed. Deze aggregatie lijkt het gevolg te zijn van veranderingen in de activiteit coefficient van het enzym. Hoge phosphaat concentratie, NAD^+ en hoge temperatuur bevorderen associatie. Het brengen van het enzym in een meer polaire omgeving veroorzaakt eveneens dissociatie. Dioxaan en 2-chloorethanol werden gebruikt om de dielectrische constante van de buffer oplossing te verlagen. Inactivering en dissociatie van het enzym is tijdsafhankelijk. Hoge phosphaat concentratie bevordert de denaturatie en inactivatie van het enzym in dioxaan en 2-chloorethanol.

Dissociatie van het enzym gaat gepaard met verlies van activiteit en vermindering van het schijnbaar α-helix gehalte. ORD en CD resultaten tonen dit aan, echter kan de mogelijkheid dat deze vermindering het gevolg is van veranderingen in vorm en grootte van het eiwit molecuul niet worden uitgesloten. Fluorescentie en CD proeven tonen aan dat als gevolg van dissociatie een amino zuur, een tryptophaan, in een meer polaire omgeving komt. Door het enzym met dioxaan te behandelen wordt eveneens een tryptophaan verschoven.

Dissociatie van het enzym kan eveneens worden bewerkstelligd door het enzym met natrium-dodecylsulfaat te behandelen. Hydrophobe en ionogene interacties zijn waargenomen. Binding van natrium-dodecylsulfaat of Tween 80 op de hydrophobe bindingsplaats heeft geen invloed op de activiteit en het absorptiespectrum van het enzym in het zichtbare gebied. Binding op de ionogene bindingsplaats veroorzaakt verlies van activiteit en geeft veranderingen in het absorptiespectrum. Gebaseerd op de pH en ionsterkte afhankelijkheid is de conclusie getrokken dat een groep, $BH^+ \rightleftharpoons B + H^+$, met een pK waarde rond pH 6.6, is betrokken bij de binding. Bij hoge natriumdodecylsulfaat concentraties wordt de binding van FAD aan het enzym verzwakt en na lange tijd staan wordt het flavine afgesplitst.

Dimerisatie van het enzym wordt bevorderd door NAD^+. Binding van NAD^+ aan het enzyme veroorzaakt veranderingen in het absorptiespectrum. Met behulp van deze spectrale titratie curves zijn twee paren NAD^+ bindingsplaatsen berekend, de bindingsplaats met de hoogste affiniteit, $K_{diss} = 35\ \mu M$, is toegeschreven aan de regulerende bindingsplaats terwijl de bindingsplaats met $K_{diss} = 90–110\ \mu M$ is toegeschreven aan de katalytische bindingsplaats. Als gevolg van de binding van NAD^+ op de regulerende bindingsplaats wordt één proton per FAD vrijgemaakt. Vergelijking van de pH-activiteit curves met computer modellen laat zien dat de activerende werking van NAD^+ in de lipoaat activiteit kan worden verklaard met een verschuiving van de pK waarde van een groep van pH 6,2 naar pH 4,9–5,0 door NAD^+ binding. Met waarnemingen

uit de literatuur suggeren deze resultaten dat de pK waarde van een SH-groep wordt verschoven door binding van NAD^+ aan het enzym. Deze groep wordt verondersteld functioneel te zijn in het active centrum van het enzym in de S^--vorm.

REFERENCES

ATKINSON, M. R., DIXON, M. and THORBER, J. M., Biochem. J., **82** (1962) 29 P.

BENESCH, R. E., BENESCH, R. and MACDUFF, G., Biochemistry, **3** (1949) 1132.

BEYCHOK, S., In Poly-α-Amino Acids, Editor: G. Fasman, Marcel Dekker, New York (1967) p. 764.

BRADY, A. L. and BEYCHOK, S., J. Biol. Chem., **244** (1969) 4634.

BRAUN, P. E. and RADIN, N. S., Biochemistry, **8** (1969) 4310.

BRESTERS, T. W., KRUL, J., SCHEEPENS, P. and VEEGER, C., FEBS-Letters, to be published, 1972.

VAN DEN BROEK, H. W. J., SANTEMA, J. S. and VEEGER, C., Eur. J. Biochem., **24** (1971) 55.

CARVER, J. P., SHECHTER, E. and BLOUT, E. R., J. Am. Chem. Soc., **88** (1966) 2562.

CASOLA, L., BRUMBY, P. E. and MASSEY, V., J. Biol. Chem., **241** (1966) 4977.

CASOLA, L. and MASSEY, V., J. Biol. Chem., **241** (1966) 4985.

CHANNING, E. P., EBERHARD, A., GUINDON, A. H., RAPLAR, C., MASSEY, V. and VEEGER, C., Biol. Bull., **123** (1962) 480.

CHILSON, O. P., COSTELLO, L. A. and KAPLAN, N. O., Biochemistry, **4** (1965) 271.

COHN, M. L., and MCMANUS, I. R., Fed. proc., **30** (1971) 1167.

COHN, M. L., WANG, L., SCOUTEN, W. and MCMANUS, I. R., Biochim. Biophys. Acta, **159** (1968) 182.

COLEMAN, D. L., and BLOUT, E. R., In Conformations of Biopolymers, Editor: G. N. RAMACHANDRAN, Academic Press, New York (1967) p. 123.

CONSTANTINIDES, S. M. and DEAL, W. C., J. Biol. Chem., **245** (1970) 246.

CONWAY, A. and KOSHLAND, D. E. Jr., Biochemistry, **7** (1968) 4011.

COSANI, A., PEGGION, E., VERDINI, A. S. and TERBOJEVICH, M., Biopolymers, **6** (1968) 963.

COWGILL, R. W., Arch. Biochim. Biophys., **104** (1964) 84.

COWGILL, R. W., Biochim. Biophys. Acta, **120** (1966) 196.

D'ANNA, J. A. and TOLLIN, G., Biochemistry, **10** (1970) 57.

DETERMANN, H., Gelchromatographie, Springer Verlag, Berling-Heidelberg-New York (1967).

DIXON, M. and WEBB, E. C., Enzymes, Longmans, London, 1964.

EDMUNDSON, D. E. and TOLLIN, G., Biochemistry, **10** (1970) 124.

FASMAN, G. D., Methods in Enzymology, vol. **V1** (1963) p. 928.

FASMAN, G. D., Bodenheimer, E. and Lindblow, C., Biochemistry, **3** (1964) 1665.

FINDLAY, D., MATHIAS, A. P. and RABIN, B. R., Biochem. J., **85** (1962) 139.

FISH, W. W., REYNOLDS, J. A. and TANFORD, C., J. Biol. Chem., **245** (1970) 5166.

FÖRSTER, T., Ann. Physik, **2** (1947) 55.

FRIDOVIC, I., J. Biol. Chem., **238** (1963) 592.

GAWRONSKI, T. H. and WESTHEAD, E. W., Biochemistry, **8** (1969) 4261.

GOEDDE, H. W., GRAFEN, P. and SCHMIDT, U., Biochem. Zeitschr., **339** (1963) 23.

GOLDMAN, D. S., Biochim. Biophys. Acta, **45** (1960) 279.

GRANDE, H. and MÜLLER, F., Personal communication, 1972.

GREENFIELD, N., DAVIDSON, B. and FASMAN, G. D., Biochemistry, **6** (1967) 1631.

GUNSALUS, I. C., Fed. Proc., **13** (1954) 715.

HAAKER, H., BRESTERS, T. W. and VEEGER, C., FEBS-Letters, to be published 1972.

HAGER, L. P. and GUNSALUS, J. C., J. Am. Chem. Soc., **75** (1953) 5767.

HAYAKAWA, T., SAKARIA, Y., AIKAWA, T., FUKUYOSHI, Y. and KOIKE, M., in Proc. 2nd Conference on Flavins and Flavin Enzymes, editor K. YAGI, Tokyo University Press, Tokyo (1968) p. 99.

HENNING, U., Sonderdrück aus Hoppe-Seyler's Zeitschrift für Physiologische Chemie, **353** (1972) 501.

HILVERS, A. G., VAN DAM, K. and SLATER, E. C., Biochim. Biophys. Acta, **85** (1964) 206.

VON HIPPEL, P. H. and SCHLEICH, T., in Structure and Stability of Biological Macromolecules, Editors: S.N. TIMASHEFF and G. D. FASMAN, Marcel Dekker, New York 1969 p. 417.

IIZUKA, E. and TSI YANG, J., Biochemistry, **4** (1965) 1249.
KALSE, J. F. and VEEGER, C., Biochim. Biophys. Acta, **159** (1968) 244.
KAWAHARA, K., KIRSHNER, A. G. and TANFORD, C., Biochemistry, **4** (1965) 1203.
KAWAHARA, Y., MISAKA, E. and NAKANISHI, Y., J. Biochem., **60** (1968) 77.
KLOTZ, I. M., Arch Biochem. Biophys., **9** (1946) 109.
KOIKE, M., REED, L. J. and CAROLL, W. R., J. Biol. Chem., **235** (1960) 1924.
KOIKE, M., REED, L. J. and CARROLL, W. R., J. Biol. Chem. **238** (1963) 30.
DE KOK, A., SPENCER, R. D. and WEBER, G., Fed. Proc., **27** (1968) 298.
LASKOWSKI, M. Jr., LEACH, S. J. and SCHERAGA, H. A., J. Am. Chem. Soc., **82** (1960) 571.
LEVINE, W., GUIDITTA, A. and ENGLARD, D. J., Neurochem., **6** (1960) 28.
LISTOWSKY, I., ENGLARD, S., RETHEIL, J. J. and SEIFTER, S., Biochemistry, **5** (1966) 2548.
LUMRY, R. and BILTONEN, R., in Structure and Stability of Biological Macromolecules, Editors: S.N. TIMASHEFF and G. D. FASMAN, Marcel Dekker, New York 1969 p. 65.
MASSEY, V., Biochim. Biophys. Acta, **30** (1958) 205.
MASSEY, V., Biochim. Biophys. Acta, **38** (1960) 447.
MASSEY, V., The Enzymes, Acad. Press, New York, 1963 vol. **7**, p. 275.
MASSEY, V., GIBSON, Q. and VEEGER, C., Biochem. J., **77** (1960) 341.
MASSEY, V., HOFMANN, T. and PALMER, G., J. Biol. Chem., **237** (1962) 3820.
MASSEY, V. and PALMER, G., J. Biol. Chem., **237** (1962) 2347.
MASSEY, V. and VEEGER, C., Biochim. Biophys. Acta, **40** (1960) 184.
MASSEY, V. and VEEGER, C., Biochim. Biophys. Acta, **48** (1961) 33.
MATTHEWS, J., and REED, L. J., J. Biol. Chem., **238** (1963) 1869.
MAYHEW, S. G., MÜLLER, F. and MASSEY, V., Personal communication, 1972.
MCCORMICK, D. B., Experientia, **26** (1970) 243.
MCKENZIE, H. A., SAWYER, W. H. and SMITH, M. B., Biochim. Biophys. Acta, **147** (1967) 73.
MILES, D. W. and URRY, D. W., Biochemistry, **7** (1968) 2791.
MILES, D. W. and URRY, D. W., Biochemistry, **7** (1968) 404.
MISAKA, E., J. Biochem., **60** (1966) 496.
MISAKA, E. and NAKANISHI, K., J. Biochem., **53** (1963) 465.
MISAKA, E. and NAKANISHI, K., Biochem. J., **58** (1965) 436.
MISAKA, E. and NAKANISHI, K., J. Biochem., **58** (1965) 1.
MISAKA, E. and NAKANISHI, K., J. Biochem., **60** (1966a) 17.
MISAKA, E. and NAKANISHI, K., J. Biochem., **59** (1966b) 545.
MISAKA, E. and NAKANISHI, K., J. Biochem., **60** (1968) 17.
MOFFITT, W., J. Chem. Phys., **25** (1956) 467.
MOFFITT, W. and YANG, J. R., Proc. Natl. Acad. Sci. U.S., **42** (1956) 596.
MÜLLER, F., HEMMERICH, P., EHRENBERG, A., PALMER, G. and MASSEY, V., Eur. J. Biochem., **14** (1970) 185.
NAWA, H., BRADY. W. T., KOIKE, M. and REED, L. J., J. Am. Chem. Soc., **82** (1960) 896.
NISSELBAUM, T. S. and BODANSKI, O., J. Biol. Chem., **236** (1961) 617.
OLSON, J. A. and ANFINSEN, C. B., J. Biol. Chem., **202** (1953) 841.
PALMER, G. and MASSEY, V., Biochim. Biophys. Acta **58** (1962) 349.
PITTZ, E. P., and BELLO, J., Arch. Biochem. Biophys., **147** (1971) 284.
RABINOWITCH, B., Arch. Biochem. Biophys., **124** (1968) 258.
RAY, A., REYNOLDS, J. A., POLET, H. and STEINHARDT, J., Biochemistry, **5** (1966) 2606.
REED, L. J., KOIKE, M., LEVITCH, M. E. and LEACH, F. R., J. Biol. Chem., **232** (1959) 143.
REED, L. J. and OLIVER, R. M., Brookhaven Symposia in Biology, **21** (1968) 397.
REYNOLDS, J. A., GALLAGER, J. P. and STEINHARDT, J., Biochemistry, **9** (1970) 1232.
REYNOLDS, J. A., HERBERT, S., POLET, H. and STEINHARDT, J., Biochemistry, **6** (1967) 937.
REYNOLDS, J. A. and TANFORD, C., Proc. Natl. Acad. Sci., **66** (1970a) 1002.
REYNOLDS, J. A. and TANFORD, C., J. Biol. Chem., **245** (1970b) 5161.
SAKURAI, Y., HAYAKAWA, T., FUKUYOSHI, Y. and KOIKE, M., J. Biochem., **65** (1969) 313.
SAVAGE, N., Biochem. J., **67** (1957) 146.
SEARLS, R. L., Fed. Proc., **19** (1960) 36.
SEARLS, R. L. and SANADI, D. R., J. Biol. Chem., **235** (1960) PC 32.

SEARLS, R. L. and SANADI, D. R., J. Biol. Chem., **235** (1960) 2485.
SEARLS, R. L., and SANADI, D. R., J. Biol. Chem., **236** (1961) 580.
SHAPIRO, A. L., VINUELA, E. and MAIZEL, J. V. Jr., Biochim. Biophys. Res. Commun., **28** (1967) 815.
SHIREY, T. and HUANG, Ru Chih C., Biochemistry, **8** (1969) 4138.
SIMPSON, R. T. and VALLEE, B. L., Biochem. Biophys. Res. Com., **22** (1966) 712.
SLUYTERMAN, L. A. A. E., Biochim. Biophys. Acta, **139** (1967) 418.
SMITH-JOHANNSEN, H. and DRYSDALE, J. W., Biochim. Biophys. Acta, **194** (1969) 43.
STAAL, G. E. J. VOETBERG, H. and VEEGER, C., unpublished results.
STARK, G. R., STEIN, W. H. and MOORE, S., J. Biol. Chem., **236** (1961) 436.
STEIN, A. M. and CZERLINSKI, G., Fed. Proc., **26** (1967) 842.
STEIN, A. M. and STEIN, J. H., Biochemistry, **4** (1965) 1491.
STEIN, A. M. and STEIN, J. H., Personal communication, 1972.
STOCKELL, A., J. Biol. Chem., **234** (1959) 1286.
STRAUB, F. B., Biochem. J., 33 (1939) 787.
STRITTMATTER, P., J. Biol. Chem., **236** (1961) 2329.
SU, G. and WILSON, J. E., Arch. Biochem. Biophys., **143** (1971) 253.
SUND, Pyridine Nucleotide-Dependent Dehydrogenases, Springer verlag, Berlin, 1969, EISENBERG, H., p. 293.
SWAISGOOD, H. E. and TIMASHEFF, S. N., Arch. Biochem. Biophys., **125** (1968) 344.
SWANEY, J. B. and KLOTZ, I. M., Biochemistry, **9** (1970) 2570.
TANFORD, C., Physical Chemistry of Macromolecules, Wiley, New York 1961.
TANFORD, C., Advances in Protein Chemistry, vol. **17** (1962) p. 69.
TANFORD, C., J. Mol. Biol. **39** (1969) 539.
TANFORD, C., Advances in Protein Chemistry, vol. **24** (1970) p. 1.
THEORELL, H., Proc. 4th Intern. Congr. Biochem., Vienna (1958).
THEORELL, H. and NYGAARD, A. P., Acta Chem. Scand., **8** (1954) 1649.
TIMASHEFF, S. N., SUSI, H., TOWNEND, R., STEVENS, L., GORBUNOFF, M. J. and KUMOSINSKI, T. F., In Conformations of Biopolymers, Editor: G. N. RAMACHANDRAN, Academic Press, New York (1967) p. 173.
TIMASHEFF, S. N., TOWNEND, R. and MESCANTI, L., J. Biol. Chem., **241** (1966) 1863.
TRENDHAM, D. R., Biochem. J., **109** (1968) 603.
URRY, D. W. and JI, T. H., Arch. Biochem. Biophys., **128** (1968) 802.
VEEGER, C. and MASSEY, V., Biochim. Biophys. Acta, **37** (1960) 181.
VEEGER, C. and MASSEY, V., Biochim. Biophys. Acta, **64** (1962) 83.
VEEGER, C. and MASSEY, V., Biochim. Biophys. Acta, **67** (1963) 679.
VEEGER, C., DERVARTANIAN, D. V., KALSE, J. F., DE KOK, A. and KOSTER, J. F., in SLATER, E. C., Symp. on Flavins and Flavo-proteins, B.B.A. Library, vol. 8, p. 242, Elsevier, Amsterdam 1966.
VEEGER, C. and VISSER, J., Methods in Enzymology, editors D. B. MCCORMICK and L. D. WRIGHT, vol. **xv111** part B (1971) p 582.
VEEGER, C., VOETBERG, H., PRONK, J. and VISSER, A. J. W. G., in Conference on Redox EnZymes, Editors: Å. ÅKESON and A. EHRENBERG, Pergamon Press, Oxford, 1970, in press.
VEEGER, C., VOETBERG, H., VISSER, J., STAAL, G. E. J. and KOSTER, J. F. In Flavins and Flavoproteins, Editor: H. KAMIN, University Park Press, Baltimore 1971, p. 261.
VISSER, J., Studies on Lipoamidedehydrogenase, Ph. D. Thesis. Mededelingen van de Landbouwhogeschool, Veenman, Wageningen (1970).
VISSER, J. and VEEGER, C., Biochim. Biophys. Acta, **206** (1970) 224.
VISSER, J. VOETBERG, H. and VEEGER, C., In Pyridine Nucleotide-Dependent Dehydrogenases, Editor: H. SUND, Springer Verlag, Berlin-Heidelberg-New York, 1969 p. 359.
WALAAS, E. and WALAAS, O., Acta Chem. Scand., **10** (1956) 122.
WEBER, G., J. Opt. Soc. Am., **46** (1956) 962.
WEBER, G. and YOUNG, L., J. Biol. Chem., **239** (1964) 1424.
WEBER, K. and OSBORN, M., J. Biol. Chem., **224** (1969) 4406.
WILLIAMS, C. H. and ARSCOTT, L. D., Personal communication, 1972.

WILLIAMS, C. H., BURLEIGH, B. D., RONCHI, R., ARSCOTT, L. D. and JONES, T., in Flavins and Flavoproteins, editor H. KAMIN, University Park Press, Baltimore, (1971) p 295.
WILSON, J. E., in Flavins and Flavoproteins, editor: H. KAMIN, University Park Press, Baltimore, (1971) p 133.
WREN, A. and MASSEY, V., Biochim. Biophys. Acta, **110** (1965) 329.
YANG, J. T., *In Conformations of Biopolymers*, Editors: RAMACHANDRAN, G. N., Academic Press, New York (1967) p. 157.

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