BBA 4054

SOME PHYSICO-CHEMICAL PROPERTIES OF SULPHITE-MODIFIED UREASE

L. W. NICHOL* AND J. M. CREETH**
Department of Physical and Inorganic Chemistry, University of Adelaide (South Australia)

(Received October 15th, 1962)

SUMMARY

Urease (EC 3.5.1.5) in buffered sulphite solutions sedimented as a single boundary in the ultracentrifuge. An analysis of the boundary shape showed that the material was essentially homogeneous in terms of a sedimentation coefficient distribution, indicating that, in the environmental conditions reported, sulphite effectively reversed the polymerisation observed earlier, but did not affect the structural integrity of the monomer. In contrast, marked heterogeneity was apparent on subjecting certain samples to electrophoresis: the data strongly suggested that the unsymmetrical scission of S–S bonds by sulphite introduced–SSO_3^- groups onto the monomer. Moreover, a cyclic mechanism was indicated, involving oxygen as an essential constituent, whereby urease in the presence of sulphite may be converted to an electrophoretically homogeneous modified form with a high mobility. Additional sedimentation-velocity experiments confirmed the function of dissolved oxygen in this respect, and in the formation of polymers in sulphite-free solutions.

INTRODUCTION

In an earlier paper[1], it was shown that urease (EC 3.5.1.5) tends to aggregate in solution forming a series of well-defined polymers; by the action of sulphite, all polymeric components are converted to a form possessing the same sedimentation coefficient as the monomer, and the enzymic activity is simultaneously increased. These findings indicated that polymerisation occurs by the formation of intermolecular disulphide (S–S) bonds, the reaction with sulphite being due to the scission of these bonds. It was inferred that the polymers are much less active enzymically, whereas the sulphite-modified monomer is highly active.

Since many kinetic studies have been performed[2–4] on urease in the presence of sulphite and other S-nucleophilic bases, the characterisation of the sulphite-modified enzyme is important and further studies of the system have therefore been made. This paper deals with three questions raised in the earlier work. (a) Since sulphite breaks all intermolecular S–S bonds, some action on intramolecular S–S bonds might be anticipated, leading to partial cleavage or disruption of the monomer. Although

---

[*] Present address: Department of Physical Biochemistry, Australian National University, Canberra, Australia.

[**] Present address: Lister Institute of Preventive Medicine, London S.W. 1 (Great Britain).

only one component could be found in the ultracentrifuge at pH 7–7.5, this is not a sensitive test, and accordingly the sedimentation coefficient distribution has been measured at pH 7.1. (b) Because the S–S bond is broken to yield one $-SSO_3H$ and one –SH group[5,6], the monomer produced by sulphite action should be heterogeneous; electrophoresis experiments have been made to verify this prediction and to indicate the extent of modification. (c) Intermolecular S–S bonds could arise either by atmospheric oxidation of –SH groups or by mercaptan–disulphide interchange[7]. In order to decide between these alternatives, the effect of dissolved oxygen has been studied under a variety of conditions.

EXPERIMENTAL

Materials

Enzyme preparations: Urease Sample 6 described previously[1] and two new preparations (Samples 8 and 9), made by a similar procedure, were used in this work. The new samples (from a different batch of jack-bean meal) were found to contain protein impurities of low electrophoretic mobility; these impurities were removed quantitatively by preparative electrophoresis. Ultracentrifugal analysis before and after this step showed that the impurities were responsible for the slowly-sedimenting species (~ 4S) previously observed in several preparations of urease. Cysteine hydrochloride was omitted from all stages of preparation of Sample 9, which was stored in solution at 2–4° in the absence of any protecting agent. The precautions previously taken to avoid metal–ion contamination were followed.

The variability of specific activity found[2] among different preparations prohibits the use of activity measurements alone to determine concentrations; accordingly all samples were analysed in the ultracentrifuge to identify the species present and to estimate the relative proportions. As before, total protein concentrations were determined in separate refractometric experiments.

Buffers: Analytical grade materials were used; details of composition and pH are given in the text. In one experiment, an oxygen-free environment was required. In this case, purified nitrogen was passed through the boiling buffer solution, which was subsequently cooled in a dry box under nitrogen. Dialysis, with several changes of buffer, was carried out in the dry box, and the ultracentrifuge cell was filled with the enzyme solution in the same environment.

Solutions were generally dialysed in the cold for 7 days before examination in the ultracentrifuge or by electrophoresis, no special precautions being taken to eliminate oxygen.

Methods

Electrophoresis and sedimentation: Previous practices[1] were followed. The method of BALDWIN[8] was used in the determination of the sedimentation-coefficient distribution: in this method, one obtains an apparent (differential) distribution function, $g^*(\mathfrak{s})$ for the range of $\mathfrak{s}$ encountered (*cf.* ref. 9, p. 764 et seq.). The quantity $\mathfrak{s}$ is a reduced coordinate having the same dimensions as the sedimentation coefficient, s, and is defined by the equation

$$\mathfrak{s} = [\ln (r/r_0)]/\omega^2 t$$

where r is the radial distance to the point in question, r_0 the value of r at the meniscus,

ω the angular velocity and t the time. The apparent distribution function (for the same value of r) is given by

$$g^* (\mathfrak{s}) = \omega^2 t\, r^3(\partial n_c/\partial r)/r_0^2 n_c^0$$

where n_c is the difference in refractive index of solution and solvent, and n_c^0 is the initial value of n_c before sedimentation commences. In this way, curves of $g^* (\mathfrak{s})$ against $\mathfrak{s}$ were determined for various times during the sedimentation experiments. The apparent distribution $g^* (\mathfrak{s})$ reduces to the true distribution $g(s)$ when anomalies due to diffusion, and to concentration-dependence of s, are eliminated. Several procedures are available, none of which is free from disadvantages. For the case of urease, it was found that the curves of $g^*(\mathfrak{s})$ against $(\mathfrak{s})$ were nearly symmetrical, and the range of $\mathfrak{s}$ decreased progressively with time; the latter characteristic indicates that diffusion is a major factor in the boundary spreading. Accordingly the extrapolation method of BALDWIN[10] was adopted. The quantity $(\mathfrak{s} - \bar{\mathfrak{s}})^2$ was determined at values of the ratio $g^* (\mathfrak{s})/g^*(\mathfrak{s})_{\max}$ equal to 0.2, 0.4, 0.6, and 0.8 for both sides of the peak. The parameter $\bar{\mathfrak{s}}$, the weighted mean of the distribution of $\mathfrak{s}$[8] was calculated in each case from the distribution curve; values thus obtained were virtually identical with those found from the maximum of the gradient curve or from the square root of the second moment[11]. Three experiments at different initial protein concentrations were treated in this way, four exposures in each experiment being analysed.

RESULTS

Sedimentation

Sedimentation velocity experiments on sulphite-treated urease were carried out at pH 7.1. In phosphate buffer (pH 7.1) the earlier investigation had shown the presence of a 19-S component alone: the ionic strength used in this work was 0.27, for direct comparison with the work of SUMNER, GRALÉN AND ERIKSSON-QUENSEL[12]. This observation was confirmed in the present study. In some experiments at pH 7.1 at lower ionic strengths, small proportions (reaching 5 % in some cases) of a 12-S component have been observed. Conditions under which this component increases in proportion have been studied, and will be the subject of another communication; in the present paper we shall be concerned only with conditions under which the 12-S component is not a complicating factor. The patterns obtained were subjected to detailed analysis, with the purpose of detecting the presence of components different from the 19-S, but not resolved as separate peaks.

The apparent distributions of sedimentation coefficient were calculated, and used to obtain values of $(\mathfrak{s} - \bar{\mathfrak{s}})$ as a function of time (t); then graphs of $(\mathfrak{s} - \bar{\mathfrak{s}})^2$ were plotted against $e^{-\bar{\mathfrak{s}}\omega^2 t}/t$ where ω is the angular velocity. Both leading and trailing sides of the peak were analysed, but corresponding values of $(\mathfrak{s} - \bar{\mathfrak{s}})^2$ were virtually identical. Fig. 1a shows the graph for the most concentrated solution investigated; the other solutions gave analogous results. Fig. 1b shows the curves for a particular value of $g^*(\mathfrak{s})/g^* (\mathfrak{s})_{\max}$ at different concentrations. The extrapolations to infinite time (zero value of the abscissa) are indicated by the dashed lines; since the experimental points do not lie on straight lines, the extrapolations are to this extent uncertain, but there can be no doubt that all values of $(\mathfrak{s} - \bar{\mathfrak{s}})$ are close to zero at infinite time. The behaviour closely resembles that of hog thyroglobulin[13]. The urease used in these experiments (Sample 6) had shown approx. 30 % of polymers prior to treatment with sulphite. Thus sulphite-modified urease does not exhibit any measurable heterogeneity at pH 7.1 and $I = 0.27$ in terms of sedimentation coefficient.

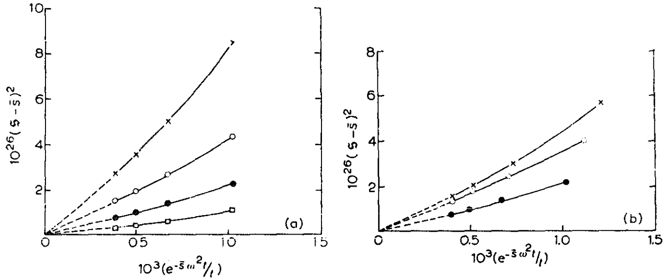

Fig. 1. Time dependence of the apparent sedimentation coefficient distribution for sulphite-treated urease. (a) Experiment at a concentration of 0.4 %: curves refer to $g^*(s)/g^*(s)_{max}$ values of 0.2 (×—×), 0.4 (○—○), 0.6 (●—●) and 0.8 (□—□), on the trailing side of the boundary. (b) Experiment at a concentration of: 0.4 % (●—●), 0.2 % (○—○) and 0.1 % (×—×); $g^*(s)/g^*(s)_{max} = 0.6$ in each case.

Electrophoresis

Experiments were carried out on urease at pH 7.5, both with and without sulphite, at ionic strengths of 0.10 and 0.05. The mobilities and apparent proportions of the various components found are shown in Table I, which also records the ultracentrifugal analyses. Typical electrophoretic patterns are shown in Fig. 2. At $I = 0.10$, the mobility recorded for the untreated sample (Expt. 1) agrees closely with the earlier value found for this pH[1]. In the presence of sulphite (Expt. 2) the original single component almost completely disappears and two new components are found; these are appreciably faster, but are not completely resolved from one another, nor from the small amount of the component possessing approximately the mobility of the untreated material.

TABLE I

EFFECT OF SULPHITE ON THE ELECTROPHORETIC PROPERTIES OF UREASE AT pH 7.5

The mobility values, referring to 1°, and electrophoretic (EP) proportions were obtained from measurements on descending boundaries and apply to the components labelled in Fig. 2; the ultracentrifugal (UC) classification follows that of Table II of CREETH AND NICHOL[1]. Veronal–chloride buffer was used throughout. Expt. 2a was made on the solution recovered from Expt. 2 after periodic oxygenation over 14 days.

Expt. No.	*Sample No.*	*Sulphite concentration (M)*	*I*	*Mobilities (cm²/sec V $\times 10^5$)*			*Proportions (%) EP*			*UC*		
				1	*2*	*3*	*1*	*2*	*3*	*19 S*	*28 S*	*36+ S*
1	9	0.0	0.10	−6.8	—	—	100	0	0	47	27	26
2	9	0.03	0.10	−7.1	−9.3	−9.8	20	55	25	100	0	0
3	8	0.0	0.05	−8.3	—	—	100	0	0	82	13	5
4	8	0.013	0.05	−8.4	−9.8	—	25	75	0	100	0	0
2a	9	0.03	0.10	−7.1	—	−9.8	20	0	80	100	0	0

At $I = 0.05$ (Expts. 3 and 4), similar behaviour was observed, with two minor differences. Firstly, the untreated sample gave a peak which showed appreciable heterogeneity (Fig. 2c); this contrasts with the single symmetrical peak found at $I = 0.10$ (Fig. 2a). This is expected because of the high polymer content and the fact that electrophoretic resolution is increased at lower ionic strength[14,15]. It was shown previously that observable electrophoretic heterogeneity would not be expected at $I = 0.10$. The weight-average mobility at $I = 0.05$ agrees within 3 % of that expected from the mobility at $I = 0.10$, after applying the correction described by ABRAMSON, MOYER AND GORIN[16]. In the presence of sulphite at $I = 0.05$ (Fig. 2d), only two recognisable components were found, of which the faster was a new species. Again, the resolution from the slower component was incomplete. The mobility of the new component, after correction to $I = 0.10$, becomes $-8.3 \cdot 10^{-5}$ cm²/sec V. This differs appreciably from the values found for any component at $I = 0.10$, so the new species cannot be identified with any produced at the higher ionic strength.

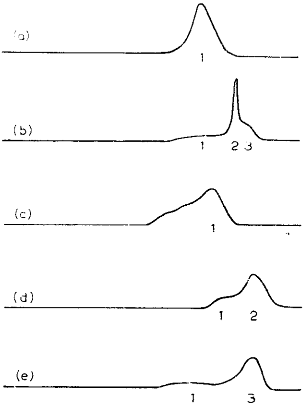

Fig. 2. Electrophoretic patterns for urease under the conditions described in Table I: the patterns at $I = 0.1$ were obtained 4–6 h at 2.5 V/cm, those at $I = 0.05$ after 2–3 h at 5 V/cm; patterns (a) through (e) correspond to Expts. 1, 2, 3, 4 and 2a of Table I, respectively.

The mobility values, and particularly the electrophoretic proportions shown in Table I are subject to unavoidably large errors (due to the low urease concentrations employed), and are therefore only approximate. Nevertheless, they are sufficient to show that the faster electrophoretic species cannot be produced solely from the polymerised fraction of the original urease: in Expt. 3, for example, the ultracentrifugal analysis showed that only 20 % of the enzyme was present in polymeric form, whereas the electrophoretic analysis of Expt. 4 indicated that about three-quarters of the sulphite-treated material had a higher mobility than the original. The results at higher ionic strength are similar in this respect, and it must therefore be inferred that part of the monomer can be converted into the faster electrophoretic species.

The effect of oxygen on urease solutions

The apparent conversion of urease monomer to the more mobile form by sulphite, and the phenomenon of polymer formation in sulphite-free solutions, may both be interpreted (see below) in terms of the direct reaction of -SH groups with dissolved oxygen. The effect of oxygen on the following systems was therefore investigated: (a) native urease, free of protecting agent (b) sulphite-modified enzyme after removal of sulphite (c) sulphite-modified enzyme in the continued presence of sulphite. The ultracentrifuge results obtained in this part of the investigation are shown in Table II.

The first two experiments in Table II refer to native urease solutions, and show that an appreciable proportion of monomer is converted to polymers by 24-h contact with oxygen; the experiment was prolonged over a further three days, but it was then found that all components specified had precipitated, and the solution was not enzymically active.

TABLE II

EFFECT OF OXYGEN ON UREASE SOLUTIONS AT pH 7.5 AND $I = 0.10$

Expts. 1 and 2 refer to separate portions of cysteine-free urease solution (Sample No. 9) while Expts. 4 and 5 were performed on portions of the solution obtained in Expt. 3.

Expt. No.	*Treatment*	*Ultracentrifugal proportions (%)*		
		19 S	*28 S*	*36+ S*
1	None	47	27	26
2	Saturated with oxygen, stood 24 h	37	27	36
3	Na_2SO_3 (0.03 M) stood 7 days	100	0	0
4	Na_2SO_3 removed by dialysis against oxygen-saturated buffer over 3 days	85	12	3
5	Na_2SO_3 removed by dialysis against oxygen-free buffer over 3 days	100	0	0

The remaining results in Table II refer to sulphite-treated materials. They show that, after the removal of sulphite, dimers and trimers of urease may be formed extensively from the modified monomer when oxygen is present; no polymerisation occurs, however, when oxygen is excluded.

The effect of oxygen in the presence of sulphite was determined by electrophoretic measurements in addition to ultracentrifugal analysis. A typical result is recorded in Table I (Expt. 2a). As expected, polymerisation does not occur, but the component of intermediate electrophoretic mobility observed before oxygenation is no longer present; instead, at least 80 % of the protein moves as a sharp symmetrical peak with a mobility corresponding to the fastest component previously observed (Fig. 2e). It is therefore concluded that oxygen, in the presence of sulphite, converts most of the urease to a fairly homogeneous modified form with a high mobility.

DISCUSSION

In the calculations of the sedimentation-coefficient distributions, the extrapolation procedure to infinite time eliminates the effect of diffusional spreading, but makes no explicit allowance for concentration-dependence effects. This is a recognised disadvantage of the method, but is not a serious limitation on the result, provided that the experiment is conducted at a concentration where s is within 10 % of $s°$ (see ref. 10). This condition was fulfilled even for the most concentrated solution used; the more dilute solutions showed no trends indicative of greater heterogeneity. The results therefore show that virtually all intermolecular S–S bonds are broken at pH 7.1 and $I = 0.27$, while structurally significant S–S bonds are unaffected (compare CECIL AND WAKE[17]).

The minor electrophoretic heterogeneity found in polymer-containing urease solutions at low ionic strength (Fig. 2c) may be ascribed to the differences in frictional coefficient between the polymers, the charge of the monomer being largely conserved in the polymerisation process. In contrast with this situation, the more marked heterogeneity of sulphite-treated urease (free of polymers) can only be ascribed to differences in charge between molecules whose frictional coefficients are virtually identical. This behaviour may be interpreted, and correlated with the ultracentrifugal results, on the basis of the mechanism proposed by BAILEY AND COLE[18] for the reaction of protein S–S bonds with sulphite in the presence of an oxidizing agent. In the present context, the relevant reactions are:

$$\mathrm{RSSR} + \mathrm{SO_3}^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{RS}^- + \mathrm{RSSO_3}^- \tag{1}$$

$$\mathrm{RS}^- + \mathrm{H}^+ \leftrightharpoons \mathrm{RSH} \tag{2}$$

$$2\mathrm{RSH} + [\mathrm{O}] \rightarrow \mathrm{RSSR} + \mathrm{H_2O} \tag{3}$$

Thus a pathway is provided for the conversion of native monomer to the modified form $\mathrm{RSSO_3}^-$; the possibility of sulphite action on a structurally unimportant S–S bond in the monomer cannot, however, be excluded.

In the presence of sulphite, and when oxygen is not specifically excluded, the presence of a variety of monomeric species would be anticipated, depending on the number of S–S bonds involved in dimerisation, the direction of the unsymmetrical scission and the extent of Reaction 3. This agrees with the observed electrophoretic behaviour (Expts. 2 and 4 in Table I) the new components of higher mobility than the original being identified with monomeric urease in which $-\mathrm{SSO_3}^-$ groups have been partially substituted for the –SH originally present; at pH 7.5, the former group is ionized but the latter is not[19]. With excess oxygen, Reaction 3 becomes important, and a greater degree of conversion to the fully-sulphited form (of highest mobility) would be expected. This is realised in practice (Expt. 2a in Table I). The complete conversion of RSH to $\mathrm{RSSO_3}^-$ would not be anticipated on a more detailed model, since the introduction of $-\mathrm{SSO_3}^-$ groups in the neighbourhood of a susceptible –SH group must lessen the probability of dimerisation.

The role of oxygen as an essential constituent of the polymerisation process, suggested by the electrophoretic results, is confirmed by the ultracentrifugal analyses of preparations from which the sulphite was removed (Table II). This is substantial evidence in favour of the direct oxidation mechanism rather than that involving mercaptan–disulphide interchange. The relative stability of urease solutions reported previously is explained by the presence of the protecting agent cysteine.

The observation that sulphite-treated urease gives a single peak in the ultracentrifuge is not itself sufficient to show that Reaction 1 goes far to the right, since a monomer-dimer system in rapid equilibrium gives a single peak also[20,21]. However, the sedimentation-coefficient distribution of sulphite-treated urease is centered about the 19-S component alone, and the sedimentation coefficient–concentration curve has a negative slope[1]; these findings indicate that rapid equilibrium does not occur. The observation that the electrophoretic boundaries do not resolve completely is therefore attributed to the presence of a range of components rather than to equilibrium.

The most probable interpretation of the results is that the equilibrium position for Reaction 1, under the conditions of pH and sulphite concentration used, lies far to the right, and that the velocity constant k_{-1} is rather small; the latter would be expected from the large negative entropy of activation. Reaction 3 will be slow for a similar reason; it must be noted that this reaction takes precedence over the oxidation of sulphite to sulphate.

The electrophoretic mobility values found for the various components allow a rough calculation of the number of $-SSO_3^-$ groups which have been substituted for $-SH$ in the reaction with sulphite. The limitations on the accuracy of this type of calculation have been discussed previously[1,22], but the urease system is one where major errors are not anticipated. Using the same molecular parameters as before[1], one obtains −47 for the charge of the native urease monomer at pH 7.5. The faster species (Components 2 and 3 in Table I and Fig. 2) give the values −62 and −65 respectively. These values are all increased by about 10 % if a small degree of molecular asymmetry is assumed; the differences are accordingly largely independent of the model used. These figures imply that about 20 $-SH$ groups on the monomer may be converted to $-SSO_3^-$ by way of preliminary S–S bond formation. Incomplete conversion would result in the presence of a large number of differently modified species, possessing very small differences in mobility; this could well account for the incomplete resolution observed. HELLERMAN, CHINARD AND DEITZ[23] reported about 22 active $-SH$ groups in urease which reacted with *p*-chloromercuribenzoate without appreciable loss of enzymic activity; these groups may reasonably be identified with those involved in $-SSO_3^-$ substitution.

ACKNOWLEDGEMENT

We wish to thank General Motors-Holden's Ltd. for a Post-Graduate Fellowship, held by L.W.N.

REFERENCES

[1] J. M. CREETH AND L. W. NICHOL, *Biochem. J.*, 77 (1960) 230.

[2] G. B. KISTIAKOWSKY, P. C. MANGELSDORF, A. J. ROSENBERG AND W. H. R. SHAW, *J. Am. Chem. Soc.*, 74 (1952) 5015.

[3] J. F. AMBROSE, G. B. KISTIAKOWSKY AND A. G. KRIDL, *J. Am. Chem. Soc.*, 72 (1950) 317.

[4] G. B. KISTIAKOWSKY AND W. E. THOMPSON, *J. Am. Chem. Soc.*, 78 (1956) 4821.

[5] H. T. CLARKE, *J. Biol. Chem.*, 97 (1932) 235.

[6] J. W. H. LUGG, *Biochem. J.*, 26 (1932) 2144.

[7] C. HUGGINS, D. F. TAPLEY AND E. V. JENSEN, *Nature*, 167 (1951) 592.

[8] R. L. BALDWIN, *J. Am. Chem. Soc.*, 76 (1954) 402.

[9] J. W. WILLIAMS, K. E. VAN HOLDE, R. L. BALDWIN AND H. FUJITA, *Chem. Rev.*, 58 (1958) 715.

[10] R.L. BALDWIN, *J. Phys. Chem.*, 63 (1959) 1570.

[11] R. J. GOLDBERG, *J. Phys. Chem.*, 57 (1953) 194.

[12] J. B. SUMNER, N. GRALEN AND I. ERIKSSON-QUENSEL, *J. Biol Chem.*, 125 (1938) 37.

[13] I. J. O'DONNELL, R. L. BALDWIN AND J. W. WILLIAMS, *Biochim. Biophys. Acta*, 28 (1958) 294.

[14] H. SVENSSON, *Arkiv Kemi*, 22 (1946) 10.

[15] J. R. CANN, *J. Am. Chem. Soc.*, 71 (1949) 907.

[16] H. A. ABRAMSON, L. S. MOYER AND M. H. GORIN, *Electrophoresis of Proteins*, Reinhold, New York, 1942.

[17] R. CECIL AND R. G. WAKE, *Biochem. J.*, 82 (1962) 401.

[18] J. L. BAILEY AND R. D. COLE, *J. Biol. Chem.*, 234 (1959) 1733.

[19] R. CECIL AND J. R. MCPHEE, *Advan. Protein Chem.*, 14 (1959) 255.

[20] G. A. GILBERT, *Discussions Faraday Soc.*, 20 (1955) 68.

[21] G. A. GILBERT, *Proc. Roy. Soc. (London) Ser. A*, 250 (1959) 377.

[22] J. M. CREETH AND D. J. WINZOR, *Biochem. J.*, 83 (1962) 566.

[23] L. HELLERMAN, F. P. CHINARD AND V. R. DEITZ, *J. Biol. Chem.*, 147 (1943) 443.