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Purification and Properties of Cytochrome c_3 of *Desulfovibrio salexigens**

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ABSTRACT: Cytochrome c_3 of *Desulfovibrio salexigens* was purified to homogeneity as judged by disc gel electrophoresis and by ultracentrifugation. The molecular weight calculated from sedimentation-diffusion data is 13,387 and from amino acid composition it is 13,901. It has a partial specific volume of 0.72 ml/g, an isoionic point of 10.8, a redox potential of -205 ± 5 mV at pH 7.0, and contains three heme groups per mole of protein. *D. salexigens* cytochrome c_3 differs from the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* in its amino acid composition and electrophoretic mobility on polyacrylamide gel at pH 6.6. Immunodiffusion data demonstrate that it does not share a common precipitating antigenic determinant with the cytochrome c_3 of *D. desulfuricans* or *D. vulgaris*.

As part of a program on the comparative biochemistry of the nonsporulating, sulfate-reducing bacteria, we have conducted studies to determine if the differences in the deoxyribonucleic acid base composition (Saunders *et al.*, 1964) of the physiologically related species of *Desulfovibrio* (Postgate and Campbell, 1966) would be reflected in differences in the cytochrome c_3 of these organisms. This protein was chosen because of its small size and its role in the metabolism of these organisms (Postgate, 1956, 1965).

In previous papers we have examined the properties of the cytochrome c_3 of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris*. The cytochromes c_3 of these two species are similar in their redox potentials, sedimentation constants, molecular weights, partial specific volumes, and polypeptide chain lengths, and contain three heme groups per mole of protein (Drucker and Campbell, 1969; Drucker *et al.*, 1970a). They differ, however, in their isoionic points, amino acid compositions (Drucker *et al.*, 1970a), optical rotation properties (Drucker *et al.*, 1970b), and electrophoretic properties, and do not share a common precipitating antigenic determinant (Drucker and Campbell, 1969).

LeGall *et al.* (1965) and Bruschi-Heriaud and LeGall (1967) have reported that the cytochrome c_3 of *Desulfovibrio gigas* has some properties in common with the cytochrome c_3 of *D. vulgaris* but differs from the latter in its electrophoretic behavior and amino acid composition. By use of the data of LeGall *et al.* (1965) and Bruschi-Heriaud and LeGall (1967) and a molecular weight of 13,425 we have calculated that *D. gigas* cytochrome c_3 also contains three hemes per mole of protein (Drucker *et al.*, 1970a). *D. gigas* cytochrome c_3 has been shown not to cross-react with antisera against the cytochrome c_3 of *D. desulfuricans*, *D. vulgaris*, or *D. salexigens* (E. Trousil and L. L. Campbell, unpublished data).

* From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801. *Received March 12, 1970.* This investigation was supported by Public Health Service Grant AI-04673 from the National Institute of Allergy and Infectious Diseases. Presented in part at the 66th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif. April 25–May 5, 1966 (Drucker and Campbell, 1966).

† Taken in part from the dissertation presented to the Graduate Faculty of the University of Illinois in partial fulfillment of requirements for the Ph.D. degree (1967). Predoctoral trainee (GM-510) of the National Institute of General Medical Sciences, U. S. Public Health Service during the tenure of this work.

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This paper describes the purification and properties of the cytochrome c_3 of *Desulfovibrio salexigens* (Postgate and Campbell, 1966) and shows that it is significantly different from the cytochrome c_3 of *D. desulfuricans* and *D. vulgaris*.

Materials and Methods

The organism used was *D. salexigens*, strain British Guiana (NCIB 8403)—DNA base composition of 45.6% guanine and cytosine. This strain was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, through the courtesy of J. R. Postgate and J. M. Shewan.

Stock cultures were maintained in Baars' (1930) medium supplemented with yeast extract (0.1% w/v) and NaCl (2.5% w/v.) For large-scale cultivation, *D. salexigens* was grown in a medium containing, per liter of deionized water: KH_2PO_4, 0.5 g; NH_4Cl, 1.0 g; Na_2SO_4, 2.6 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; sodium lactate, 6.0 g; yeast extract (Difco), 1.0 g; and NaCl, 2.5 g. The medium was adjusted to pH 7.2 and autoclaved. Just prior to inoculation, filter-sterilized $Fe(NH_4)_2(SO_4)_2$ was added to a final concentration of 10 μg/ml. This medium is a modification (Baker *et al.*, 1962) of the medium C of Butlin *et al.* (1949) supplemented with NaCl.

The organism was grown in 12-l. florence flasks containing 9 l. of modified medium C. A 10% (v/v) inoculum was used. Anaerobiosis was obtained with pyrogallol plugs made alkaline with a solution containing K_2CO_3 (15% w/v) and NaOH (10% w/v). After incubation for 24–36 hr at 30° the cells from 120-l. cultures were harvested with a Sharples centrifuge. The cells were stored at −20° until used for the isolation of cytochrome c_3.

Amberlite CG-50 resin was prepared for chromatography as described by Drucker and Campbell (1969). Dowex 1-X8 (200–400 mesh, spherical, in the chloride form) was prepared for chromatography as follows: The resin (100 g) was suspended in 1 l. of distilled water, stirred for 15 min, and allowed to settle 15 min before decantation. This step was repeated (4–5 times) until there were no fines in the supernatant fluid after 15-min settling. The material was then washed with 1 l. of 4 N NH_4OH, and the excess NH_4OH was removed by four washes with distilled water (15-min settling time between each wash). The resin was then washed with 1 l. of 4 N HCl and then four times with 1 l. rinses of distilled water or until there was a negative chloride reaction with 1% (w/w) silver nitrate solution. The resin was suspended in 1 l. of 2 M ammonium acetate and stirred for 15 min. This step was repeated four times (15-min settling periods) or until the effluent was pH 5.5–6.0. The material was then washed four times with distilled water and stored in distilled water.

Ammonium sulfate (enzyme grade) was obtained from Mann Research Laboratories. All other chemicals and reagents were purchased from commercial sources and were of the highest purity available.

The methods employed by Drucker and Campbell (1969) were used to determine the following properties: redox potential, electrophoretic behavior on polyacrylamide gel, and immunological reactivity.

Sedimentation–velocity studies were conducted as described by Drucker and Campbell (1969). The cytochrome was dissolved in the appropriate solvent and dialyzed against 1 l. of the corresponding solvent overnight at 5°. Three protein concentrations were employed to determine concentration dependence of the sedimentation constant. Centrifugations were run in a single-sector synthetic boundary cell.

Amino acid composition was determined by the procedures used by Drucker *et al.* (1970a) except that the Beckman-Spinco amino acid analyzer was equipped with a microcuvet and a scale expander. Amino acid composition was calculated by the mole ratio method on duplicate samples hydrolyzed at 110° for 24, 48, and 72 hr. The amide content was determined by a modification of the method of Hirs *et al.* (1954) as described by Drucker (1967).

The isoionic point and partial specific volume were calculated from the amino acid composition (Drucker *et al.*, 1970a).

Heme content was determined from extinction measurements, iron analysis, and from the alkaline pyridine hemochrome as described by Drucker *et al.* (1970a).

Cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* were purified to homogeneity according to Drucker and Campbell (1969).

Results

Purification of D. salexigens Cytochrome c_3. The purification procedure employed depended upon the duration of storage of the cells at −20°. Method I was used for cells stored at −20° for 6 months to 2 years. It was found, however, that the cytochrome c_3 prepared from cells stored at −20° for less than 6 months was not pure (by this method) and behaved as an acidic protein rather than as a basic protein upon polyacrylamide gel electrophoresis. Additional purification steps were necessary to obtain pure cytochrome c_3. These additional steps are given in method II.

Method I. Frozen cells (100–200 g wet weight) were thawed at 37° for 60–90 min. The cytochrome was extracted by a modification of the method described by Sels *et al.* (1965) for yeast cytochrome c. For each gram dry weight of cells, 2 ml of 8.5% (v/v) glycerol in water and 1 ml of ethyl acetate were combined and evacuated with a house vacuum line. This mixture was then added to the cells in a 500-ml erlenmeyer flask equipped with an evacuation and gassing closure. Deoxyribonuclease (10 μg) and $MgCl_2 \cdot 6H_2O$ (10 μg) were added and the suspension was stirred under Linde high-purity nitrogen for 15 min. Sodium dithionite (170 mg/g dry weight of cells) was added to the flask and the suspension was stirred under nitrogen for 12–16 hr at room temperature. NOTE: *If the suspension was not kept under these reduced conditions, the yield of cytochrome was lowered.*

The autolyzed cell suspension was diluted in 10 volumes of deionized water, stirred for 5–10 min, and centrifuged at 13,000*g* for 70 min at 5°. The brown supernatant fluid was decanted and stored in an ice bath. The pellet was suspended in 10 volumes of deionized water, stirred for 5–10 min, and centrifuged as described above. The light brown supernatant liquid was combined with the first supernatant fraction. CG-50 resin (25 mg in 0.25 ml of water per 200 mg dry weight of cells) was added and the mixture was stirred overnight at 5°. The resin was then allowed to settle 1 hr at 5° and the clear brown supernatant fraction was siphoned off.

The resin and small amount of remaining liquid was poured into a 2 × 6 cm column. The column was washed with 600–800 ml of 0.001 M ammonium phosphate buffer, pH 7.0

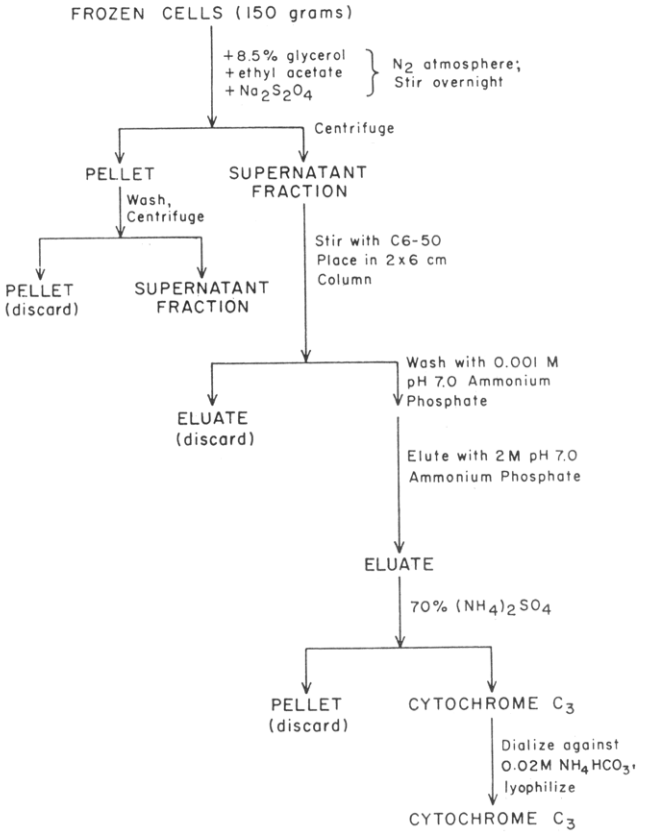

FIGURE 1: Flow diagram of the purification of *D. salexigens* cytochrome c_3 (method I).

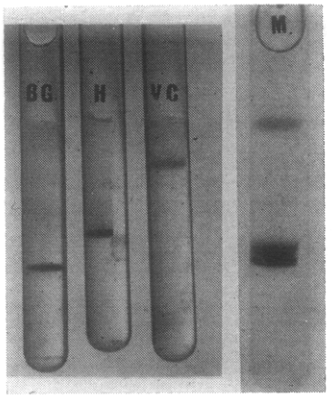

FIGURE 2: Polyacrylamide gel electrophoresis of cytochromes c_3 of *D. salexigens*, *D. vulgaris*, and *D. desulfuricans* at pH 6.6. *D. salexigens* (BG) and *D. vulgaris* (H) cytochrome c_3 run at 25 μg per column; *D. desulfuricans* (VC) at 15 μg. M is a mixture of all three cytochromes run on the same column. The gels were stained with sudan black B and destained with acetic acid.

(molarity with respect to ammonium ion as will be the case with all ammonium phosphate buffers used in this study). The cytochrome was eluted with 2 M ammonium phosphate buffer, pH 7.0.

The cytochrome fraction was brought to 70% saturation by the slow (60–90 min) addition of solid ammonium sulfate and then centrifuged at 39,000*g* for 30 min. NOTE: *If the eluate from the CG-50 column was dialyzed before the addition of ammonium sulfate, almost all of the cytochrome precipitated at 70% saturation, thereby reducing the yield and purity of the material obtained.*

The supernatant fluid from the above step was decanted and dialyzed against 6 l. of 0.02 M NH_4HCO_3 for 3 days with six changes of NH_4HCO_3. The material was lyophilized, taken up in a minimal amount of distilled water, and lyophilized; this step was repeated three times.

Figure 1 outlines the method I purification procedure. With this procedure 30–60 mg of cytochrome c_3 with a purity index of 2.2–2.4 was obtained from 1 kg wet weight of cells. The purity index was calculated by the method of Horio and Kamen (1961). The cytochrome preparation gave a single band upon polyacrylamide gel electrophoresis.

Method II. To obtain pure cytochrome c_3 from cells of *D. salexigens* stored less than 6 months at $-20°$, a modification of method I was required.

The lyophilized cytochrome obtained in the last step of method I was dissolved in a minimal amount of 0.01 M Tris-HCl buffer, pH 8.1. This solution was brought to 90% saturation by the slow (45–60 min) addition of solid ammonium sulfate. After standing for 30 min the suspension was centrifuged at 3020*g* for 5 min at 5°.

The supernatant fraction was dialyzed 12 hr against two changes (1 l. per change) of 0.01 M Tris-HCl buffer, pH 8.1. The dialyzed material was then concentrated under pressure (40 psi) in a Model 50 Amicon cell (UM-10 membrane) to a volume of 5 ml. The volume was brought up to 30 ml by the addition of 0.02 M ammonium bicarbonate and concentrated to 5 ml as above. The concentrated material was stored for 1 week at $-20°$ and thawed. The precipitate was removed by centrifugation at 3020*g* for 5 min. The supernatant fluid was passed through a Dowex 1-X8 column (2 $\times$ 10 cm) and the cytochrome was lyophilized as described in method I.

D. salexigens cytochrome c_3 prepared by method II had the same purity index and the same electrophoretic and immunological properties as that described by method I from cells stored at $-20°$ for longer than 6 months. The yield of pure cytochrome ranged from 15 to 30 mg per kg wet weight of cells.

Properties of D. salexigens Cytochrome c_3. Figure 2 shows that the cytochromes c_3 of *D. salexigens*, *D. desulfuricans*, and *D. vulgaris* differ in their electrophoretic mobility on polyacrylamide gel at pH 6.6. It is evident that *D. salexigens* cytochrome c_3 is more basic than the other two cytochromes. If the cytochromes are run on the same gel (Figure 2M) the order of mobility remains the same and there is no interaction between the three types of cytochromes.

The order of electrophoretic mobilities is in agreement with the isoionic points calculated from amino acid composition data. The isoionic point of *D. salexigens* cytochrome c_3 is 10.8 compared to isoionic points of 10.0 and 7.2 for the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans*, respectively (Drucker *et al.*, 1970a).

The redox potential (-205 ± 5 mV at pH 7.0) of *D. salexigens* cytochrome c_3 is identical with the redox potential of the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* (Drucker and Campbell, 1969).

Based upon extinction measurements ($\epsilon^{\text{reduced}}_{552\text{ nm}}$ 76 cm^{-1}/μmole per ml) *D. salexigens* cytochrome c_3 has a heme content of 2.71 moles/mole of protein. This value is based on the extinc-

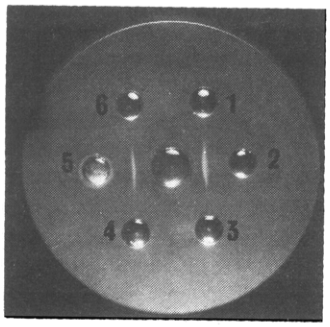

FIGURE 3: Immunological specificity of *D. salexigens* cytochrome c_3 as judged by Ouchterlony immunodiffusion. Center well contains antiserum against *D. salexigens* cytochrome c_3. Wells 1 and 6 contain *D. desulfuricans* cytochrome c_3; wells 2 and 5 contain *D. salexigens* cytochrome c_3; wells 3 and 4 contain *D. vulgaris* cytochrome c_3 (all cytochromes c_3 at 50 μg/well). Note reaction only with the homologous antigen.

tion of mammalian type cytochrome c ($\epsilon^{\text{reduced}}_{552\text{ nm}}$ 28 cm^{-1}/μmole per ml), a protein of approximately the same molecular weight containing 1 mole of heme/mole of protein (Margoliash and Schejter, 1966). *D. salexigens* cytochrome c_3 has an iron content of 1.31%. On the basis of the iron content of cytochrome c (0.45%) and a molecular weight of 12,056 for *D. salexigens* apocytochrome c_3 this gives a heme content of 2.83 moles/mole of protein. The alkaline pyridine hemochrome gave a $\epsilon^{\text{reduced}}_{550\text{ nm}}$ value of 78.8. The heme content was estimated using the $\epsilon^{\text{reduced}}_{550\text{ nm}}$ (29.1 cm^{-1}/μmole per ml) of heme c and gave a value of 2.71. Thus by three methods the heme content of this cytochrome c_3 is about 3 moles/mole of protein. This is the same value reported for the cytochromes c_3 of *D. desulfuricans*, *D. vulgaris*, and *D. gigas* (Drucker *et al.*, 1970a).

An immunological comparison was made between the cytochromes c_3 of *D. salexigens*, *D. desulfuricans*, and *D. vulgaris*. *D. salexigens* cytochrome c_3 reacts with the antiserum prepared against it (Figure 3) but does not react with antisera prepared against the cytochrome c_3 of either *D. desulfuricans* or *D. vulgaris*. Figure 3 also shows that the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* do not react with the antiserum against *D. salexigens* cytochrome c_3. These data demonstrate that *D. salexigens* cytochrome c_3 does not share a common precipitating antigenic determinant with the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris*. We have previously shown that there is no immunological cross-reaction between the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* (Drucker and Campbell, 1969).

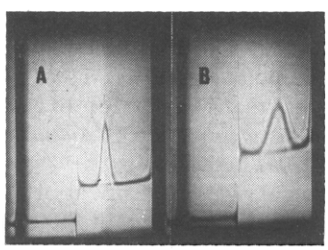

FIGURE 4: Sedimentation velocity pattern of *D. salexigens* cytochrome c_3 at 59,780 rpm. The cytochrome (7 mg/ml) was dissolved in 0.01 M sodium phosphate buffer, pH 7.6. Direction of sedimentation is to the right. Time after attaining speed was (A) 8 min (bar angle 50°); (B) 32 min (bar angle 40°).

TABLE I: Amino Acid Composition of *D. salexigens* Cytochrome c_3.[a]

Residue	Moles/Mole of Protein	
	b	*c*
Lysine	22.44	22
Histidine	6.35	6
Arginine	0	0
Aspartic acid	8.56	9
Threonine	6.45	6
Serine	11.70	12
Glutamic acid	5.12	5
Proline	5.37	5
Glycine	10.92	11
Alanine	12.97	13
$^1/_2$-Cystine	7.59	8[d]
Valine	6.11	6
Methionine	2.15	2
Isoleucine	1.94	2
Leucine	3.65	4
Tyrosine	1.00	1
Phenylalanine	3.16	3
Tryptophan	0	0[e]
Total number of residues		115
Molecular weight of apoprotein		12,056
Add 3 hemes		1,845
Molecular weight		13,901

[a] Corrections were made for the destruction of serine, threonine, and methionine by extrapolation to zero time of hydrolysis. [b] Moles/mole of protein as calculated from amino acid analysis. Recovery of amino acid residues (g/100 g of protein) was 99.94%. [c] Moles/mole of protein to nearest whole integer. [d] Determined as cysteic acid and carboxymethylcysteine. [e] See Drucker *et al.* (1970a) for method employed for tryptophan determination.

Table I gives the amino acid composition of *D. salexigens* cytochrome c_3 as calculated by the mole ratio method. No tryptophan or amide was detected by the methods employed. The total number of amino acid residues and the molecular weight calculated from the amino acid analysis are not significantly different from the values reported by Drucker *et al.* (1970a) for the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris*.

The partial specific volume calculated from the amino acid composition was 0.72 ml/g compared to 0.72 and 0.73 ml per g for the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans*, respectively (Drucker *et al.*, 1970a).

Figure 4 shows the sedimentation velocity pattern of *D. salexigens* cytochrome c_3 in 0.01 M sodium phosphate buffer, pH 7.6. The $s_{20,w}$ was calculated to be 3.29 Svedberg units (S). Since this value is significantly higher than the values reported by Drucker and Campbell (1969) for the cytochromes c_3 of

TABLE II: Amino Acid Residues of Cytochrome c_3 of *Desulfovibrio* Species.

	Type of Cytochrome c_3			
Residue	*D. salexigens*[a]	*D. vulgaris*[b]	*D. desulfuricans*[b]	*D. gigas*[c]
	Moles/Mole of Protein			
Lysine	22	17	14	17
Histidine	6	8	7	8
Arginine	0	1	0	0
Aspartic	9	12	7	17
Threonine	6	5	4	5
Serine	12	5	5	5
Glutamic	5	6	11	4
Proline	5	6	9	4
Glycine	11	10	8	12
Alanine	13	10	10	9
$^1/_2$-Cystine	8	8	8	8
Valine	6	7	10	8
Methionine	2	3	1	0
Isoleucine	2	0	1	4
Leucine	4	2	6	4
Tyrosine	1	2	3	1
Phenylalanine	3	2	4	2
Tryptophan	0	0	0	1
Total number of residues	115	105	106	109

[a] Data from this study. [b] Data from Drucker *et al.* (1970a). [c] Data from Bruschi-Heriaud and LeGall (1967).

D. desulfuricans (1.86 S) and *D. vulgaris* (2.02 S) we thought it might represent an aggregated form of *D. salexigens* cytochrome c_3. We, therefore, examined the effect of NaCl on the sedimentation velocity of this cytochrome c_3. Figure 5 shows the sedimentation pattern of *D. salexigens* cytochrome c_3 in 0.01 M sodium phosphate buffer–0.43 M NaCl, pH 7.6. In this solvent the $s_{20,w}$ was calculated to be 1.81 S. Runs were made in both solvents at three protein concentrations and the respective sedimentation constants were independent of cytochrome concentration over a range of 0.4–1.3% (w/w). All plots of log x *vs.* time were linear. The marked difference in S values between the two solvents suggests that *D. salexigens* cytochrome c_3 aggregates (or forms polymers) at low salt concentrations. More detailed studies will be carried out to examine this effect.

Using the value of 1.81 S, and values for the diffusion constant and partial specific volume of 12.4×10^{-7} cm^2/sec (Drucker and Campbell, 1969) and 0.72 ml/g, respectively, a molecular weight of 13,387 was calculated by the Svedberg equation. This value is in excellent agreement with the value of 13,901 calculated from the amino acid composition (Table I). These values are also similar to the molecular weights reported by Drucker and Campbell (1969) and by Drucker *et al.* (1970a) for the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris*.

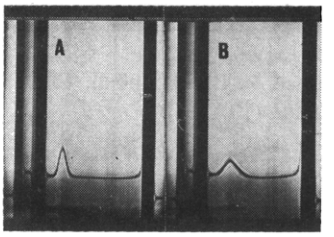

FIGURE 5: Sedimentation velocity pattern of *D. salexigens* cytochrome c_3 at 59,780. The cytochrome (4.6 mg/ml) was dissolved in 0.01 M sodium phosphate buffer–0.43 M NaCl, pH 7.6. Time after attaining speed was: (A) 8 min; (B) 32 min, bar angle 60°.

Discussion

The data presented here and in previous studies (Drucker and Campbell, 1969; Drucker *et al.* 1970a; LeGall *et al.*, 1965; Bruschi-Heriaud and LeGall, 1967) show that the cytochromes c_3 of *D. salexigens*, *D. desulfuricans*, *D. vulgaris*, and *D. gigas* have similar molecular weights, polypeptide chain lengths, partial specific volumes, redox potentials, and number of heme groups. They differ significantly, however, in their electrophoretic mobilities, isoionic points, immunological specificities, and amino acid compositions. Thus, they appear to be structurally different proteins which carry out similar functions. We have previously suggested that these differences may be the result of conservative amino acid substitutions in the cytochromes c_3 of these organisms (Drucker and Campbell, 1969; Drucker *et al.*, 1970a). Such substitutions, in turn, could account for the observed differences in immunological reactivity and electrophoretic behavior of these proteins. The amino acid compositions presented in Table II tend to support these suggestions. Amino acid sequence studies now in progress by Ambler (1968) and Ambler *et al.* (1969) and by E. Trousil and L. L. Campbell (unpublished data) should yield information on this point.

Acknowledgment

We thank P. P. Slonimski for suggesting the method used for the initial extraction of *D. salexigens* cytochrome c_3 and Connie Schimbor for excellent technical assistance.

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Binding of Guanosine Triphosphate and Adenosine Triphosphate by Rabbit Muscle Adenosine Monophosphate Deaminase*

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ABSTRACT: Equilibrium binding studies show that crystalline adenosine monophosphate deaminase from rabbit back muscle possesses two binding sites for the allosteric effector guanosine triphosphate (GTP), and a minimum of four binding sites for the allosteric effector adenosine triphosphate (ATP). Observed dissociation constants are comparable to the concentration of each effector required to produce half-maximal modification of catalytic activity under the same conditions. Binding of ATP and binding of GTP are mutually inhibitory. Treatment of the enzyme with 6.5 molar equiv of *p*-mercuribenzoate selectively abolishes binding of GTP. Enzyme treated with mercurial retains its original sedimentation properties and its ability to bind ATP.

Adenosine monophosphate deaminase (AMP-aminohydrolase, EC 3.5.4.6) from various tissues has been found to be subject to allosteric regulation by GTP and ATP (Cunningham and Lowenstein, 1965; Setlow *et al.*, 1966; Setlow and Lowenstein, 1967, 1968a,b). Smiley *et al.* (1967) have recently devised a simple procedure for crystallization of a highly active form of this enzyme from rabbit back muscle. Activity is associated with a protein of approximate mol wt 270,000, the amino acid composition of which has been determined (Wolfenden *et al.*, 1968).

The present studies were undertaken to determine whether the enzyme possesses separate binding sites for ATP and GTP, their number, and their possible mutual interaction in the absence of substrate. Since regulation of a number of allosteric enzymes has been found to be sensitive to mercurials, and sulfhydryl groups have been implicated in the action of several nucleoside deaminases (see Discussion), it was also of interest to determine the effect of mercurials on catalysis and on the binding of allosteric modifiers by AMP-deaminase.

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Experimental Section

Frozen rabbit back muscle was obtained from Pel-Freez Biochemicals, Inc. Bio-Gel P-2 was obtained from Bio-Rad, Inc. Cellulose phosphate, AMP, ATP, and GTP were purchased from Sigma Chemical Co. ATP-8-^{14}C and GTP-8-^{14}C were obtained from Schwarz BioResearch, Inc.

AMP-deaminase was prepared from rabbit back muscle essentially by the published procedure of Smiley *et al.* (1967). After column chromatography on cellulose phosphate in the presence of 2-mercaptoethanol (10^{-3} M), enzyme was recovered from active fractions by addition of solid ammonium sulfate to a concentration of 300 g/l. The pooled fractions, adjusted to pH 6.50 with K_2HPO_4, were allowed to stand overnight at 0°, and the resulting precipitate was dissolved in a minimal volume of KCl (0.45 M) containing 2-mercaptoethanol (10^{-3} M) and adjusted to pH 6.50 at room temperature. Upon cooling in ice, the enzyme crystallized immediately. It was recovered by centrifugation and recrystallized twice under the same conditions. All kinetic and binding experiments were performed on samples of crystalline enzyme dissolved in ammonium succinate buffer (0.1 M, pH 6.50) containing 2-mercaptoethanol (10^{-3} M) and incubated for 1 hr at room temperature. Following incubation, excess 2-mercaptoethanol was removed from enzyme samples by column chromatography on Bio-Gel P-2 in buffer not containing mercaptoethanol. Enzyme prepared in this way was stable for at least 10 hr at room