Reconstitution of Bovine Procarboxypeptidase A-S6 from the Free Subunits†

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ABSTRACT: The three subunits I, II, and III of bovine procarboxypeptidase A separated by reversible dimethylmaleylation can reassociate to form the reconstituted complexes I + II, I + III, and I + II + III. Since the association II + III is not possible, subunit I appears to play a central role in the formation of the complex. It is suggested that subunit I possesses two independent and specific sites for the recognition of subunits II and III. The liberation of subunit I from any of the complexes was observed to increase its activability, although to a lesser extent than predicted by assays carried out with the succinylated protein. By contrast, the bound form of subunit II was activated faster than the free form. The potential activity of the bound form and the activity of the preformed endopeptidase were also higher, suggesting a conformational change induced by association. This suggestion was fully supported by the observed modifications of the heat stability and intrinsic fluorescence spectrum of the subunit resulting from association.

Two complex forms of procarboxypeptidase A are known to exist in bovine pancreas and pancreatic juice: a ternary complex (procarboxypeptidase A-S6; sedimentation coefficient ~6 S (Yamasaki et al., 1963)) composed of different subunits I, II, and III tightly linked by noncovalent bonds, and a binary complex (procarboxypeptidase A-S5; sedimentation coefficient ~5 S (Brown et al., 1963b)) in which subunit III is lacking. Subunit I has been found to be the zymogen of bovine carboxypeptidase A, whereas subunit II has more recently been identified as a chymotrypsinogen of type C (Peanasky et al., 1969; Keil-Dlouha et al., 1972). For a number of years, subunit III has been reported to be inactive. In fact, like subunit II, this subunit contains a latent active site titratable by concentrated diisopropyl fluorophosphate at 25 °C (Puigserver, 1976), but its activation by trypsin into a fully functional chymotrypsin is not possible due to structural differences, especially the deletion of essential residues in the N-terminal region of the chain (Puigserver and Desnuelle, 1975).

Bovine procarboxypeptidase A-S6 was dissociated in the past, either by a prolonged incubation at pH 10.5 (Brown et al., 1963a) or by acylation of NH_2 groups with succinic anhydride (Freisheim et al., 1967a). Both procedures are not fully satisfactory. The first yields an irreversibly denatured subunit I and a partially modified subunit II (Puigserver et al., 1972). The second yields succinylated subunits from which the acyl groups cannot be removed under nondenaturing conditions. More recently, these difficulties were largely overcome (Puigserver and Desnuelle, 1975) by using 2,3-dimethylmaleyl anhydride as the acylating agent. Nitrogen-bound dimethylmaleic groups are readily removed at pH 6.0–7.0 and 0 °C, i.e., conditions under which most proteins remain native.

The purpose of the present work was to explore the ability for the demaleylated subunits to reassociate and to investigate the effects of reassociation on their molecular and catalytic properties.

Materials and Methods

Preparation of Subunits. Procarboxypeptidase A-S6 was prepared from an acetone powder of bovine pancreas (Puigserver et al., 1972) and dissociated by dimethylmaleylation (Puigserver and Desnuelle, 1975). The three subunits were purified to homogeneity by chromatography as already reported (Puigserver and Desnuelle, 1975). Subunit I was deacylated by a 48-h incubation at pH 7.0 and 4 °C, while for subunits II and III this was achieved at pH 6.0 for 24 h. The extent of removal of the dimethylmaleyl groups from the deacylated subunits was evaluated by measuring the amount of free amino groups by use of the trinitrobenzenesulfonic acid method (Habeeb, 1966).

Enzymes and Enzyme Activities. Bovine carboxypeptidase A was obtained by a 3-h incubation at 37 °C of 10 mg/mL solutions of subunit I with 1% (w/w) of trypsin in a 50 mM potassium phosphate buffer, pH 7.5. The residual trypsin activity was inhibited by 10 mM DFP and the resulting mixture was exhaustively dialyzed against several changes of the above buffer. Porcine chymotrypsin C was prepared in this laboratory and kindly supplied by Dr. Gratecos (Gratecos and Desnuelle, 1971). Bovine trypsin (two times crystallized) was purchased from Worthington.

The peptidase activity of carboxypeptidase A was measured spectrophotometrically at 233 nm and 37 °C against 10 mM CbGlyPhe solutions in a 25 mM Tris[1]-HCl buffer, pH 7.6, containing 0.1 M NaCl. The esterase activity of the enzyme was determined potentiometrically at pH 7.5 and 25 °C using 10 mM BzGlyOPhe solutions in a 5 mM sodium Veronal buffer containing 45 mM NaCl. The chymotryptic activity of activated subunit II was also measured potentiometrically, but at pH 7.9 with 10 mM AcTyrOEt solutions containing 3% of methanol. In all cases, an enzyme unit was defined as the amount of enzyme hydrolyzing 1 μmol of substrate/min under the conditions of the assays.

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[1] Abbreviations used are: DFP, diisopropyl fluorophosphate; CbGlyPhe, carbobenzoxyglycyl-L-phenylalanine; BzGlyOPhe, hippuryl-L-phenyllactic acid; AcTyrOEt, acetyl-L-tyrosine ethyl ester; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Sedimentation Coefficient of the Free Subunits and Equimolar Mixtures.

Molecular species	Sedimentation coefficient ($s_{20,w}$) (S)
Free subunits	
I	3.9
II[a]	2.9
III	2.7
II + III	2.7
Carboxypeptidase A	3.5
Binary complexes	
I + II (procarboxypeptidase A-S5)	4.8
I + II + II[b]	4.9 and 2.8
I + III	4.6
I + III + III[b]	4.9 and 2.6
Carboxypeptidase A + II	4.7
Carboxypeptidase A + III	4.6
Ternary complexes	
I + II + III (procarboxypeptidase A-S6)	5.8
Carboxypeptidase A + II + III	5.2

[a] In a 50 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl. [b] Mixtures of I with II or III in a 1 to 2 molar ratio.

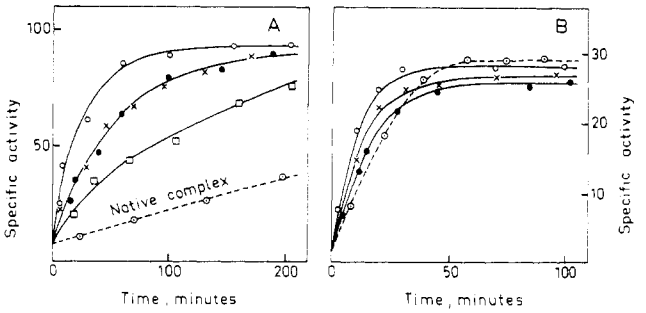

FIGURE 1: Tryptic activation of subunit I free or included in various complexes. (A) Assays without added Ca^{2+}. The solutions (0.45 mg of subunit I/mL of a 38 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl) were activated at 37 °C by 10% (w/w) of trypsin. (B) The solutions (0.1 mg of subunit I/mL of a 50 mM Tris-HCl buffer, pH 7.5) were activated at 37 °C by 3% (w/w) of trypsin in the presence of 0.125 M calcium chloride. (○ and +) Native and reconstituted procarboxypeptidase A-S6. (○ and □) Reconstituted complexes I + II and I + III. (●) Free subunit I. Activities in experiments A and B were measured against BzGlyOPhe and CbGlyPhe, respectively.

Protein Concentrations. Protein concentrations were evaluated spectrophotometrically at 280 nm taking $E_{1cm}^{1\%} = 19$ at this wavelength for all proteins except subunit I and carboxypeptidase A for which a slightly lower value, $E_{1cm}^{1\%} = 18$, was used.

Sedimentation Coefficients and Molecular Weights. Sedimentation coefficients were determined at 20 °C in an analytical Spinco-Beckman ultracentrifuge Model E equipped with temperature and speed controls. The free subunits were dissolved in a 0.1 M potassium phosphate buffer at pH 6.5. No concentration dependence was noted in the range 3.0–13.0 mg of protein/mL of buffer. Association between subunits was followed using equimolar mixtures (concentration of each subunit, $1.0–1.5 \times 10^{-4}$ M) in a 38 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl (Behnke et al., 1970).

Molecular weights were measured by sedimentation equilibrium at high and low speed (Yphantis, 1964; Van Holde and Baldwin, 1958). Partial specific volumes were experimentally determined with the aid of a carefully thermostated Parr digital microdensimeter Model DMA 02C.

Fluorescence Spectra. Solutions of the free subunits or their mixtures at the same molar concentration were made in a 38 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl. After excitation at 292 nm, the fluorescence emission was recorded at 20 °C between 300 and 400 nm with the aid of a Fica double-beam spectrofluorimeter, Model 55. The absorbance of the solutions at 280 nm never exceeded 0.02.

Results

Reconstitution of Complexes as Studied by Ultracentrifugation. The sedimentation coefficients measured using solutions of the free subunits and of their equimolar mixtures are reported in Table I. Subunit I was found by this procedure to associate with subunit II yielding a reconstituted binary complex I + II apparently similar to natural procarboxypeptidase A-S5. It also associated with subunit III leading to a hitherto unknown complex I + III. The association of the three subunits to give reconstituted procarboxypeptidase A-S6 (complex I + II + III) was possible. By contrast, no interaction could be detected between subunits II and III. It was noteworthy that subunit I cannot bind more than one subunit II or III.

Table I further shows that active carboxypeptidase A shares with its zymogen the property to bind subunits II and III. The resulting complexes (carboxypeptidase A + II and carboxypeptidase A + III) have the same sedimentation coefficient as the complexes formed with the zymogen, although the molecular weight of the enzyme is substantially lower (35 000 instead of 41 500). Therefore, the splitting of the activation peptide transforming the zymogen into an active enzyme does not abolish the sites involved in association, but probably alters the overall structure of the complex.

Similar results were obtained with the low-speed sedimentation equilibrium technique for the molecular weight of the reconstituted complex I + II + III and the native trimer (92 000 and 93 000, respectively) using the same partial specific volume value ($\bar{v} = 0.75$) for both assays. By contrast, slightly different results were obtained at high speed (89 000 and 101 000). The molecular weights determined with this latter technique for the free subunits I, II, and III were, respectively, 41 500 ($\bar{v} = 0.73$), 29 000 ($\bar{v} = 0.74$), and 27 500 ($\bar{v} = 0.74$). The sum of the three values (99 000) agrees well with the weight of the native trimer.

The equilibrium sedimentation plots (log Y vs. r^2) at high speed for both native and reconstituted procarboxypeptidase A-S6 were very close, showing that the last complex was, in fact, substantially monodisperse.

Activation of Free and Bound Subunits I and II. The time course of the tryptic activation of free and bound subunit I (esterase activity against BzGlyOPhe) in the absence of added calcium is illustrated by Figure 1A. As already reported by other authors (Yamasaki et al., 1963; Keller et al., 1958), this activation was found to be very slow in native procarboxypeptidase A-S6. It was much faster with the free or bound subunits (reconstituted complexes I + II and I + III). This latter finding suggests that the reconstituted and native trimer, although very similar, may have slightly different quaternary structures.

Figure 1B further shows that a high concentration of calcium enhances the activation of free and bound subunit I. This is especially noteworthy for native procarboxypeptidase A-S6 in which subunit I is now activated at the same rate as in the reconstituted complexes. By contrast, as shown by Figure 2, subunit II included in a complex was observed to be activated

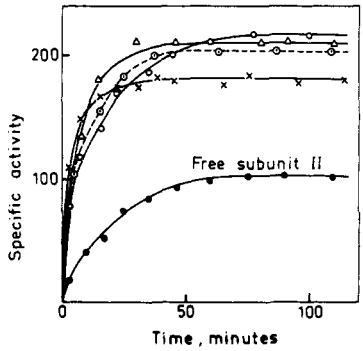

FIGURE 2: Activation of free and bound subunit II. Solutions containing 0.35 mg of subunit II/mL of a 50 mM Tris-HCl buffer, pH 8.0, were activated at 0 °C by trypsin (weight ratio, 1:200 by reference to subunit II). The specific activities against AcTyrOEt are expressed per mg of endopeptidase. Same symbols as in Figure 1, except the open triangles referring to assays in which the subunits were mixed in the order I + III + II (instead of I + II + III).

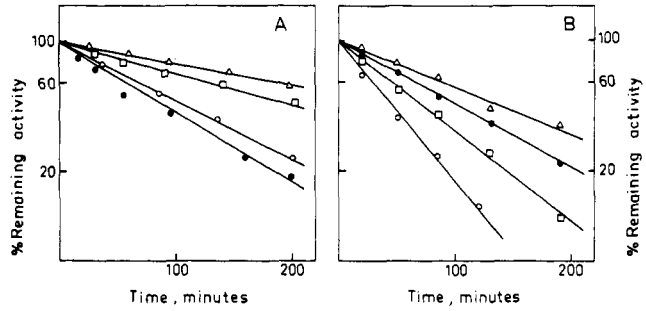

FIGURE 3: Heat stability of free subunit II and carboxypeptidase compared to that of the complexes. (A) Stabilization of subunit II dissolved (0.2 mg/mL) in the 38 mM potassium phosphate buffer, pH 7.5, containing 0.1 M KCl. The solutions were activated at 0 °C by trypsin (weight ratio 1:200) and then incubated at 40 °C for varying periods of time. (○) Free subunit II. (△) Native procarboxypeptidase A-S6. (□ and ●) Reconstituted complexes of subunit II with subunit I or carboxypeptidase, respectively. (B) Stabilization of carboxypeptidase (0.25 mg/mL) in the above buffer. After incubation at 50 °C, the remaining carboxypeptidase activity was directly measured against CbGlyPhe. (○) Free carboxypeptidase. (□, ●, and △) Respectively, reconstituted complexes of carboxypeptidase with subunit II, subunit III, or both. Endopeptidase activity (A) was determined using AcTyrOEt as substrate. Exopeptidase activity (B) was evaluated on CbGlyPhe.

much faster than the free form. Moreover, the potential specific activity AcTyrOEt of the subunit was increased 2.0–2.3-fold in the complexes. A point of interest in this respect was that the binding capacity of the endopeptidase arising from subunit II by tryptic activation was conserved and that its activity was also increased 1.7-fold. In other words, binding enhanced in this case not only the potential activity of the zymogen but also the direct activity of the preformed enzyme.

Heat Stability. The heat stability of bound subunit II and carboxypeptidase can be compared in Figure 3 with that of the free forms. Subunit II is stabilized by integration in the reconstituted complex I + II and in native procarboxypeptidase A-S6. It is not stabilized by association with carboxypeptidase. The stability of carboxypeptidase is also markedly increased by association with subunit II or/and subunit III.

Fluorescence Spectra of the Free and Bound Subunits. Intrinsic fluorescence emission spectra due to tryptophan residues in monomers and complexes after excitation at 292 nm are reproduced in Figures 4 and 5. There is a general 25-nm shift towards shorter wavelengths of the maximal emission of the proteins when compared to that yielded by free tryptophan

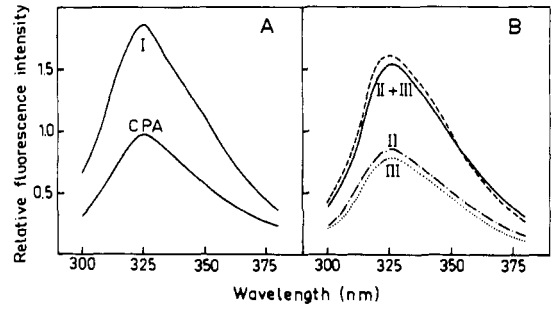

FIGURE 4: Comparison of the fluorescence spectra yielded by (A) subunit I and carboxypeptidase; (B) subunit II, subunit III, and the mixture II + III. Concentration of each protein was 2.4×10^{-7} M. The solid and interrupted lines in Figure 4B are, respectively, experimental for the mixture II + III and calculated by summation. CPA, carboxypeptidase A.

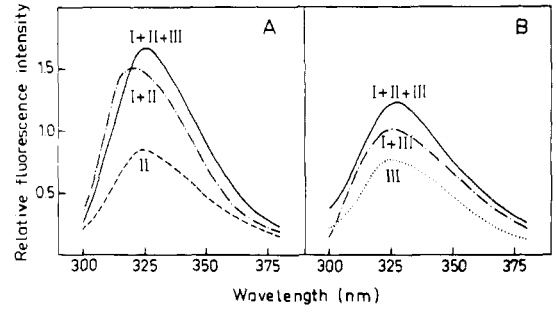

FIGURE 5: Difference between fluorescence spectra as automatically recorded by a double-beam spectrofluorimeter. (A) Case of subunit II. I + II against I and I + II + III against I + III. (B) Case of subunit III. I + III against I and I + II + III against I + II. The direct spectra of subunits II and III are also reproduced for comparison. Concentration of each protein was 2.4×10^{-7} M.

(325 nm instead of 350 nm). This very large shift (10–15 nm for most proteins (Teale, 1960; Nieto et al., 1973)) is indicative of the presence of tryptophan residues in a strongly apolar environment (Cowgill, 1967).

Moreover, Figure 4A indicates that the fluorescence intensity is significantly decreased upon conversion of subunit I into active carboxypeptidase. This decrease is not likely to result from an unequal distribution of tryptophan residues between the enzyme and the activation peptide. Subunit I and carboxypeptidase have nearly the same absorbancy at 280 nm. It more probably reflects a conformational change induced by activation. Similar variations of the fluorescence spectrum have been interpreted in terms of a modification of the protein conformation for chymotrypsinogen (Mc Clure and Edelman, 1967) and prophospholipase A (Abita et al., 1972).

In addition, the fact that subunits II and III do not interact was again confirmed by Figure 4B, showing that the fluorescence emission of this subunits mixture is practically equal to the sum of the emission yielded by each protein.

As shown by Figure 5A, the association of subunit II with subunit I or the complex I + III induced a strong increase in the intensity of fluorescence and an additional 5-nm shift of the wavelength corresponding to maximal emission. In the case of subunit III, the variations were much less (Figure 5B). The difference in the maximum fluorescence intensities of the ternary complex I + II + III in Figure 5A and 5B is due to the large effect of the association I + II.

Discussion

The dissociation of bovine procarboxypeptidase A-S6 into three different subunits I, II, and III by reversible dimethyl-

maleylation under nondenaturing conditions (Puigserver and Desnuelle, 1975) offered a unique opportunity for investigating in detail the molecular properties of the subunits, their capacity to reassociate, and the influence of reassociation on their molecular and catalytic properties. Subunit I, which is the zymogen of carboxypeptidase A, was observed to spontaneously associate with subunits II and III, yielding the two expected reconstituted dimers I + II and I + III, and also the trimer I + II + III. Dilute solutions (about 10^{-5} M) of this latter complex were found to be monodisperse, as judged by sedimentation equilibrium analysis at high speed.

However, the molecular weight of the reconstituted trimer was found to differ slightly from that of native procarboxypeptidase A-S6. In addition, the much faster activation of subunit I in the reconstituted trimer compared to that in the native complex (Keller et al., 1958) suggested a looser packing of the subunits after reassociation. A similar enhancement of subunit I activation in native procarboxypeptidase A-S5 by high Ca^{2+} concentrations has already been attributed to a loosening of the complex quaternary structure (Uren and Neurath, 1972), but it should be emphasized that calcium ions also enhance the activation of isolated subunit I (Figure 1 in this work) and that of naturally occurring monomeric procarboxypeptidases (Lacko and Neurath, 1970; Reeck and Neurath, 1972). Finally, although no dimethylmaleyl group was found attached to any amino group in subunit I, the possible modification of other amino acid residues than lysine cannot be ruled out. This last possibility might be responsible for the slightly different properties of the reconstituted complex.

Subunits II and III, which are closely related chemically (Puigserver and Desnuelle, 1975), showed no tendency to dimerize under our conditions even at high concentration. A point of interest in this respect was that they also did not associate, conferring to subunit I a central role in the formation of the complexes. In other words, subunit I is likely to possess two sites interacting with complementary regions of subunits II and III. In contrast with well-known oligomeric systems (Goldberg et al., 1966; Wood et al., 1975), these sites are likely to be largely independent. The binding capacity of one is not altered, at least qualitatively, by the saturation of the other. The two possible binary complexes I + II and I + III are formed under comparable conditions and both readily yield the complex I + II + III when mixed with the suitable subunit. Each site is also specific for a given subunit. No indication was obtained in the course of the present work consistent with the presence of twice the same subunit in a given complex. Moreover, subunit II (bovine chymotrypsinogen C) can be replaced in the association neither by bovine chymotrypsinogen B (Brown et al., 1963b) nor by porcine chymotrypsinogen C as checked in the present work.

The early finding (Brown et al., 1963b; Behnke et al., 1970) that, like subunit I, carboxypeptidase binds subunit II was confirmed and extended to subunit III, showing that none of the above-mentioned sites are destroyed upon splitting of the activation peptide, but the affinity of the sites may be lowered.

Another point of interest was that the activation of subunit I was markedly slowed down by the quaternary structure of the complexes, whereas that of subunit II was enhanced. The heat stability and activability of this latter as well as the activity of the corresponding enzyme were also increased, suggesting a conformational change due to protein–protein interaction. This suggestion was supported by spectrofluorimetry showing a substantial modification of tryptophan intrinsic fluorescence in complex I + II when compared to that in subunit II alone.

The plot of potential activity of subunit II vs. subunit I concentration led to a typical saturation curve from which an association constant certainly much better than 10^8 M^{-1} could be derived. This value illustrates well the high affinity of demaleylated subunits for each other. It is also consistent with the monodispersity of 10^{-5} M solutions of the complexes. A much lower value of only 10^4 M^{-1} was previously reported for the system carboxypeptidase–alkali-dissociated subunit II (Behnke et al., 1970). Whether this low value results from a poor efficiency of the binding site in the activated enzyme compared to that in the zymogen or from unfavorable structural modifications occurring in subunit II during alkali treatment cannot be decided at the present time. The first possibility is supported by the finding that the mixture of the subunit II with carboxypeptidase neither increased its heat stability nor the activity of the corresponding enzyme.

Although the effects exerted by association on the properties of the subunits are now better ascertained than before, the biological significance of this association in the pancreas of cattle and other species is still unclear. Subunit II can be expected to be activated first in the duodenum and hence its activability and potential activity to be markedly enhanced in the complex. Later, the conditions required for the activation of subunit I are so drastic that the endopeptidase deriving from subunits II and from subunit III are probably removed from the complex, thus conferring a second advantage to the system. However, as shown by the present work, this latter advantage is much less than suggested by previous assays carried out on the succinylated subunit (Freisheim et al., 1967b). The high association constant value mentioned above suggests that the binding of the subunits is not merely fortuitous.

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Human Hypoxanthine Phosphoribosyltransferase. Purification and Properties†

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ABSTRACT: Hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) from human erythrocytes has been purified 13 000-fold to apparent homogeneity. The native enzyme has a sedimentation coefficient of 5.9 S, determined by analytical ultracentrifugation, and a molecular weight of 81 000–83 000, determined by sedimentation equilibrium centrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates a subunit molecular weight of 26 000, suggesting that the enzyme is a trimer. Isoelectric focusing resolves three peaks of enzyme activity at pH 5.6, 5.7, and 5.9. The amino acid composition of hypoxanthine phosphoribosyltransferase is 17 Lys, 5 His, 12 Arg, 0 Trp, 31 Asx, 12 Thr, 14 Ser, 16 Glx, 14 Pro, 19 Gly, 12 Ala, 5 Cys, 18 Val, 5 Met, 11 Ile, 20 Leu, 10 Tyr, and 9 Phe. The enzyme appears to have a blocked N terminus.

Hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) is a purine salvage enzyme which catalyzes the PRPP[1]-dependent conversion of the purine bases hypoxanthine and guanine to the corresponding nucleotides IMP and GMP. This enzyme is of clinical interest since an absence of hypoxanthine phosphoribosyltransferase activity results in a severe neurological disorder, the Lesch-Nyhan syndrome (Seegmiller et al., 1967). Hypoxanthine phosphoribosyltransferase is also an important enzyme marker for the study of somatic cell genetics, because the gene is X-linked and because efficient selection systems for cells either lacking or possessing the enzyme have been developed (Szybalski et al., 1962; Thompson and Baker, 1973).

Our previous work on Chinese hamster hypoxanthine phosphoribosyltransferase (Olsen and Milman, 1974a) has been extended to the human enzyme because our current genetic studies of HeLa hypoxanthine phosphoribosyltransferase (Milman et al., 1976, 1977) and Lesch-Nyhan erythrocytes (Ghangas and Milman, 1975) require a knowledge of the characteristics of the normal human enzyme. We have adapted our procedures for purification of hypoxanthine phosphoribosyltransferase from Chinese hamster brain and liver to the purification of the enzyme from human erythrocytes. Our results indicate that human and Chinese hamster hypoxanthine phosphoribosyltransferase have similar structural properties. In addition, this procedure has yielded sufficient quantities of the purified human enzyme to enable us to perform many biochemical studies, such as amino acid analysis, N-terminal analysis, and analytical ultracentrifugation, which were not feasible with the limited amounts of enzyme which could be obtained from Chinese hamsters.

Experimental Procedures

Enzyme Purification. The procedure used was a modification of the procedure previously described for the purification of the Chinese hamster enzyme (Olsen and Milman, 1974a). All steps were performed at 0–5 °C unless otherwise specified. Protein concentration was determined by the method of Lowry (Lowry et al., 1951). Enzyme activity was measured as previously described (Olsen and Milman, 1974a). The volumes and protein concentrations at each step of the purification are given in Table I.

Crude Lysate. Ten pints of outdated human blood were obtained from the Alameda County Blood Bank. The eryth-

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[1] Abbreviations used: PRPP, 5-phosphoribosyl 1-pyrophosphate; DTT, dithiothreitol; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.